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(71) Applicant (for all designated States except US): CAN-GENE CORPORATION [CA/CA]; 26 Henlow Bay, Winnipeg, Manitoba R3Y 1G4 (CA).

(72) Inventors; and
(75) Inventors/Applicants (for US only): WOLOSKI, B., Michael, R. [CA/CA]; 8 Everden Road, Winnipeg, Manitoba R3N 3Y2 (CA). WILLIAMS, Ashley, Martin [CA/CA]; 14 Branson Crescent, Winnipeg, Manitoba R3T 5W2 (CA). SEREDA, Terrance, Jimmy [CA/CA]; #214-1720 Pembina Highway, Winnipeg, Manitoba R3T 5T4 (CA). WIEBE, Deanna, June [CA/CA]; 528 Raglan Road, Winnipeg, Manitoba R3G 3E5 (CA).

(74) Agent: BERESKIN & PARR; 40th Floor, 40 King Street West, Toronto, Ontario M5H 3Y2 (CA).

(54) Title: RHAMM PEPTIDE CONJUGATES

(57) Abstract: The present invention provides protein conjugates having a glucose-aminoglycan-targeting domain conjugated directly or indirectly to a therapeutically useful protein via chemical or peptidyl linkage. The protein conjugates selectively target certain tissues and organs and are useful for treating or preventing various physiological and pathological conditions. Methods of their use and preparation are described.

Title: RHAMM Peptide Conjugates

FIELD OF THE INVENTION

The present invention relates to drug-polypeptide conjugates and
5   methods of their use in treating or preventing various physiological and
pathological conditions. Specifically, the conjugates described herein
comprise an effective amount of one or more therapeutic agent(s)
conjugated to one or more peptide(s) or polypeptide(s) capable of binding
hyaluronan. The invention also relates to nucleic acid molecules encoding
10   such conjugates and various processes for producing such conjugates.

BACKGROUND OF THE INVENTION

Hyaluronan (hyaluronic acid or HA) is a coiled, negatively charged,
ubiquitous glucosaminoglycan comprised of repeating disaccharide units of
N-acetylglucosamine and glucuronic acid (Ogston and Stanier, 1951, 1953).
15   Circulating HA is primarily of peripheral tissue origin, and enters the blood
through the lymph (Laurent et al., 1986). In addition to its known
physicochemical functions, such as lubrication in synovial fluid in joints, HA
also performs macrostructural functions in skin, cartilage, and brain tissue
(Reed et al., 1988). HA is produced and secreted locally by chondrocytes
20   (Asari et al., 1994, Noonan et al., 1996), and is also found in connective tissue
and cartilage, comprising an integral component of the extracellular matrix,
which regulates proliferation, differentiation, and maturation of cells (Oguri
et al., 1987). Specifically, HA exerts its control of hematopoiesis at the early
progenitor stage prior to a commitment to either myeloid or lymphoid
25   lineage. In addition, HA enhances myelopoiesis and lymphopoiesis by
induction of interleukin production (Khaldoyanidi et al., 1999).
Concentration of hyaluronic acid is highest in embryonic tissue. In loose
connective tissue, it is found at a concentration of 1-3 mg/cm^3, and 0.02-1
$mg/cm3$ in vitreous humor. Due to the large solution domain of hyaluronic
30   acid, at concentrations above 1 $mg/cm3$ the molecules will become
entangled, forming a polymeric network (Comper and Laurent, 1978). This
distribution of endogenous HA should be distinguished from the
distribution of exogenously administered HA, which is subject to first pass
metabolism by the liver, and does not accumulate significantly in bone,
35   connective tissue, skin, cartilage, joints, or brain tissue (Fraser et al., 1981,

1985).

Synthesis of HA has been associated with the morphogenesis of many tissues, with wound repair, tumour invasion and cellular immune function (Boudreau et al., 1991; Iozzo, 1985; Pauli et al., 1983; Toole, 1982; Toole et al., 1989; Turley, 1984, 1992; Weigel et al., 1986, 1989). Increased levels of HA have been documented in rheumatoid arthritis, scleroderma, fibrosis of the liver, and in cancer cells (Ichida et al., 1996, Laurent et al., 1986). The underlying mechanism of action at the cellular level is believed to involve the ability of HA to elicit receptor-mediated alterations of cell motility.

High affinity HA receptors have been identified and characterized on a variety of cell types and these are namely the receptor for HA mediated mobility (or RHAMM) (Turley, E.A. et al., 1991; Hardwick, C. et al., 1992; Yang, B. et al., 1993), intercellular adhesion molecule-1 (or ICAM-1) (McCourt, P.A.G. et al., 1994) and CD44 (Underhill, C.B. et al.., 1987; Stamenkovic, I. et al., 1989; Aruffo, A. et al., 1990; Lesley, J. et al., 1990; Miyake, K. et al., 1990). Both RHAMM and CD44 have been shown to be associated with cell locomotion, cell proliferation and differentiation. Other HA-binding proteins in the extracellular milieu have also been identified and they are namely link protein, aggrecan, versican, GHAP, collagen type VI and TSG-6.

RHAMM was one of the first HA receptors to be isolated and characterized at the biochemical and molecular levels. It is an N-linked glycoprotein that binds HA with high affinity (Kd: 1 nM) and specificity. Several isoforms of RHAMM with different subcellular distribution have been identified. iRHAMM and pRHAMM are isoforms found intracellularly and on the plasma membrane, respectively, and sRHAMM is the form of a secreted protein. The molecular structure of the various RHAMM isoforms may be differentially regulated by phosphorylation and/or glycosylation status. The precise roles of the RHAMM isoforms have not been fully elucidated, but it is believed that pRHAMM and sRHAMM elicit opposite activities and the net functional behaviour of a HA-RHAMM interaction depends at least in part on the balance of pRHAMM versus sRHAMM expressed by the cells involved.

A full-length murine RHAMM cDNA has been cloned successfully

from a GT11 3T3 cDNA expression library (Hardwick, C. et al., 1992).
Immunoblot analyses of cell lysates using antibodies to peptides encoded in
the cDNA reacted specifically reacted with RHAMM protein. Using a
fragment of the clone DNA sequence, a mouse fibroblast genomic library
5   was screened to clone the genomic RHAMM gene which spans at least 20 kb
and comprises 14 exons ranging in size from 75 to 1099 bp (Entwistle, et al.,
1995).

        Similarly, a human RHAMM cDNA clone was also isolated
successfully by a combination of screening a human breast cDNA
10   expression library with the murine RHAMM cDNA as well as 5' RACE and
reverse transcription-polymerase chain reaction using messenger RNA
from the human breast cell line MCF-10A (Wang, et al., 1996). The full-
length human RHAMM cDNA encodes for a 725 amino acid protein and
shares a 85% homology with the the murine transcripts, RHAMM v4. More
15   importantly, the HA binding motif B1 - An - B2 which is shown to be critical
for the signaling capability of RHAMM is 100% conserved between human
and mouse.

        Turley in PCT application PCT/CA98/00448 published as WO
98/52590 also described the interposing of a hyaluronan binding motif
20   between a disease modifier and exogenous hyaluronan. The exogenous
hyaluronan described in this patent publication is claimed to be less than
750,000 daltons in molecular weight.

        Colony stimulating factors (CSF's) are natural hematopoietic growth
factors that regulate the production and functional activity of red blood cells,
25   white blood cells, and platelets. The most important CSF's to date are
erythropoietin (EPO), granulocyte-colony stimulating factor (G-CSF),
granulocyte-macrophage-colony stimulating factor (GM-CSF), macrophage-
colony stimulating factor (M-CSF), interleukins (IL's), and stem cell growth
factor (SCF). The continuous presence of CSF's in the concentration range
30   of 1-100 pM is required for the proliferation of hematopoietic progenitor
cells. Accordingly, CSF's can be used to restore hematopoietic dysfunction,
to augment host defense against infection, and to stimulate the proliferation
of specific cells.

        GM-CSF and G-CSF stimulate the production of granulocytes and/or
35   macrophages, extend the life expectancy of mature white cells, and

potentiate their biological activity. These white cells form the basis of the body's immune system, which may be compromised by events ranging from chemotherapy or infection to idiopathic neutropenia. In addition to reducing the duration of hospitalization, intensity of hospitalized treatment,

5   and antibiotic usage among cancer patients, both G-CSF and GM-CSF permit higher, more frequent doses of chemotherapy and improve overall prognosis. Approved and investigational therapeutic indications for GM-CSF include the treatment of the following disorders: (1) myeloid reconstitution in autologous bone marrow transplantation in non-

10   Hodgkin's lymphoma, acute lymphocytic leukemia, and Hodgkin's Disease; (2) allogenic bone marrow transplantation failure or engraftment delay; (3) acute myelogenous leukemia following high dose chemotherapy; (4) peripheral blood progenitor cell mobilization and transplantation; (5) treatment of chemotherapy-induced neutropenia (approved in Europe); (6)

15   to increase WBC counts in myelodysplastic syndrome (approved in Europe); (7) to increase WBC counts in AIDS patients receiving AZT treatment; (8) treatment of leukopenia secondary to chemotherapy; (9) treatment of neutropenia in aplastic anemia (approved in Finland); (10) prolong survival of patients with advanced melanoma (phase II/III); (11) treatment of

20   neutropenia due to hairy cell leukemia; (12) chronic lymphocytic leukemia therapy; (13) treatment of neutropenia of Felty's syndrome; (14) thermal injuries (>40% full/partial burns); (15) drug-induced neutropenia (aside from AZT and cancer chemotherapy); and (16) ganciclovir-induced neutropenia is approved in Europe.

25   Similarly, approved and investigational therapeutic indications for G-CSF include the treatment of the following disorders: (1) to decrease the incidence of infection (febrile neutropenia) in patients with non-myeloid malignancies receiving myelosuppressive cancer chemotherapy and radiation therapy; (2) treatment of severe chronic neutropenia (SCN)

30   including congenital, cyclical, or idiopathic neutropenia; (3) use in allogenic/autologous bone marrow transplantation and peripheral blood progenitor cell (PBPC) transplantation; (4) AIDS-associated neutropenia - approved in U.K; (5) acute myelogenous leukemia; (6) dose escalation of chemotherapy - Myelodysplastic syndrome; (7) severe community acquired

35   pneumonia; (8) intra-abdominal infections; (9) pneumonia/sepsis; (10)

invasive fungal infection; (11) liver transplantation; (12) neonatal infection; and (13) head injury.

Erythropioetin (EPO) is endogenously produced by the juxtatubular cells in the kidney, and to a smaller extent by the liver. The final product is a heavily glycosylated protein with 166 amino acid residues and a molecular weight of approximately 34,000D. Although EPO is not the only growth factor responsible for erythropoiesis, it is the most important stimulant for red blood cell production, by regulating proliferation of committed progenitors, maturation of erythroblasts, and release of reticulocytes into the circulation. EPO is currently approved in Canada and the USA, and is under investigation, for the treatment of conditions as follows: (1) anemia associated with chronic or progressive renal failure to improve quality of life and maintain hematocrit levels; (2) anemia associated with symptomatic HIV infection (AIDS); (3) anemia associated with non-myeloid neoplastic diseases that may be exacerbated by chemotherapy, to stimulate erythropoiesis and reduce the need for transfusion following chemotherapy; (4) autologous transfusion in patients undergoing elective surgery; (5) anemia in premature infants; (6) anemia associated with other chronic diseases (e.g. rheumatoid arthritis); (7) beta-thalassemia or sickle cell anemia; and (8) orthostatic hypertension.

Human growth hormone (hGH) is an adenohypophyseal pituitary hormonal polypeptide of 191 amino acids with an approximate molecular weight of 22,000D. The principal form of the molecule is a single chain with two intrachain disulfide linkages and no covalently bound carbohydrates. The primary function of hGH in humans is to stimulate organ and tissue growth through positive nitrogen balance and other tissue ionic constituents such as calcium, magnesium, sodium, potassium, and phosphate. hGH binds to cell-surface receptors throughout the body to enhance amino acid uptake and protein synthesis by body cells while reducing protein catabolism. hGH also exerts a number of complex effects on human lipid and carbohydrate metabolism. Briefly, hGH may be considered to be diabetogenic and switches the bodily source of fuel from carbohydrate to fat. The intracellular effects of hGH binding are primarily mediated by insulin-like growth factor 1 (IGF-1) as well as IGF-2. Long-term administration of the hormone has been documented to induce lipid

mobilization, reduce body fat stores, and increase plasma fatty acids.

hGH is currently approved and is under investigation for therapeutic use in the conditions as follows: (1) growth hormone insufficiency; (2) growth insufficiency in Turner's syndrome; (3) chronic renal insufficiency; (4) AIDS related wasting; (5) adult growth insufficiency; (6) patients with osteoporosis may benefit from the calcium; (7) retention and osteogenic effects of hGH therapy; (8) muscle wasting associated with cancer; (9) patients of short stature with Down's syndrome, nephrotic cystinosis, neural tube defects, intrauterine growth retardation, and cancer radiation treatment; (10) adjunct therapy in the treatment of catabolic conditions in burn injuries; (11) sensitization of the ovary to gonadotrophins in infertility; (12) achondroplasia; and (13) veterinary and agricultural purposes.

Interferons are potent cytokines that stimulate complex antiviral, immunomodulatory, and antiproliferative effects in response to viral infection and other biological inducers. There are three classes of interferons, namely alpha (leukocyte), beta (fibroblast), and gamma (immune), which bind their respective cell-surface receptors to enhance macrophage function, induce production of immunomodulatory proteins and cytokine receptors, and prevent viral penetration, reproduction, and protein synthesis.

Approved and investigational indications for alpha-interferon are as follows: (1) hairy-cell leukemia; (2) AIDS-related Kaposi's syndrome; (3) chronic myelogenous leukemia; (4) basal cell carcinoma; (5) multiple myeloma; (6) hepatitis; (7) juvenile laryngeal papillomatosis; (8) condylomata acuminata; (9) genital herpes; (10) cancer of the head and neck; (11) combination therapy for AIDS; (12) combination therapy in squamous cell cancer of the cervix and skin; (13) ovarian carcinoma; (14) cervical carcinoma; (15) bladder carcinoma; (16) primary malignant brain tumor; (17) renal cell carcinoma; (18) malignant melanoma; (19) esophageal carcinoma; (20) colon cancer; and (21) multiple sclerosis.

Parathyroid hormone (PTH) is an 84 amino acid polypeptide chain with a molecular weight of approximately 9500 D. The primary role of PTH is to maintain calcium concentration in the extracellular fluid, which is accomplished mainly through the activation of osteoclasts to release calcium from bone. In addition, PTH increases calcium reabsorption from the

intestinal tract and kidneys, and regulates the excretion of calcium in feces, sweat, and milk. Teriparatide, synthetic PTH available under the name Parathar, is used only as a diagnostic to differentiate between pseudohypoparathyroidism and hypoparathyroidism. Treatment of these

5 diseases is accomplished with calcium and/or vitamin D, due to greater safety with these agents. Adverse effects following teriparatide treatment include constipation, headache, loss of appetite, muscle weakness, abdominal cramping, diarrhea, nausea, tingling in the hands and feet, and urge for bowel movement.

10 Interleukins (IL's) are multifunctional cytokines which are produced by and act on the lymphopoietic system. Over 15 interleukins have been identified and are under investigation for the treatment of various pathologies, primarily cancer (Dorland's Illustrated Medical dictionary). Brief description of each are set out below:

15 IL-1 (lymphocyte-activating factor), is produced by macrophages, and induces IL-2 release from activated T cells. In the vasculature, epithelial cells produce IL-1 in order to stimulate fibroblast proliferation and enzyme and prostaglandin release.

IL-2 (T-cell growth factor) is produced by T cells following stimulation

20 by IL-1. IL-2 stimulates T cell proliferation and production of interferon-g, and is used therapeutically in the treatment of solid malignant tumors.

IL-3 (multi-CSF) is produced by activated T cells, and stimulates colony formation of hematopoietic cell lines as well as influencing eosinophil and basophil function, and resulting in pulmonary macrophage

25 proliferation. In addition to its direct effects on hematopoiesis, IL-3 acts synergistically with GM-CSF to increase blood neutrophils, eosinophils, and monocytes, and with EPO to expand the erythrocyte burst forming unit compartment and erythrocyte colony forming unit.

IL-4 (B-lymphocyte stimulating factor I) is released from activated T

30 cells. IL-4 promotes maturation and growth of T cells and mast cells and regulates B cell function. This regulation includes proliferation, antigen presentation, and immunoglobulin production.

IL-5 is also produced by activated T cells to promote differentiation of B cells and eosinophils and increases production of IgA by B cells.

35 IL-6 is released from macrophages, fibroblasts, and activated T cells

to stimulate thymocyte and B cell differentiation and immunoglobulin production.

IL-7 is produced by mesangial and epithelial stromal cells to promote early stage differentiation of lymphocytes.

5   IL-8 is released by monocytes and endothelial cells as a neutrophil chemotactic and activator.

IL-9 is a growth factor that is released from T cells and macrophages in vitro.

IL-10 inhibits the production of cytokines, increases B cell viability,
10   and induces major histocompatability II gene expression.

Several other interleukins have been identified, and await specific characterization. Specific therapeutic uses of interleukins include (Harrison's Principles of Internal Medicine): (1) IL-1, 3, and 6 improve blood counts in some patients with aplastic anemia; (2) IL-1 improves platelet and
15   granulocyte recovery following chemotherapy; (3) IL-2 is approved for the treatment of cancer, IL 4, IL-6, IL-12 are currently under investigation for this indication (University of Pittsburgh Cancer Institute); (4) clinical trials of IL-2 and IL-12 to stimulate T cells and natural killer cells in AIDS patients are underway; (5) IL-8 has been shown to induce de novo angiogenesis in a rat
20   model (Norrby, et al., 1996); (6) IL-10 decreases the severity of psoriasis (Asadullah et al., 1999); (7) IL 11 has been approved for the treatment of chemotherapy-induced thrombocytopenia (Schwertschlag et al., 1999); (8) IL-11 exhibits anti-inflammatory effects in animal models of inflammatory bowel disease, inflammatory skin disease, autoimmune joint disease, and
25   various infection-endotoxemia syndromes; IL-11 is also under investigation in the treatment of Crohn's disease, psoriasis, and rheumatoid arthritis (Schwertschlag et al., 1999); and (9) IL-12 may help control mycobacterium avium infection (NIH, 1998).

SUMMARY OF INVENTION

30   The present inventors have by their invention developed conjugates which specifically target tissues and organs enriched in endogenous HA such as the lymph nodes, bone marrow, skin and muscle. Accordingly, these conjugates can exert focussed therapeutic action(s) directly on the site of the disorder thereby allowing lower dosages and/or frequency of drug
35   administration required to achieve equivalent therapeutic efficacy as the

conventional dosages of the corresponding unconjugated therapeutic protein alone. Lower therapeutic dosages required also translates into lower immunogenicity of the conjugated protein as compared to the native protein. As a result, conjugates of the present invention would improve
5   patient compliance and reduce direct and indirect costs associated with the drug substance and its administration. Accordingly, in its broad aspsect the present invention provides a conjugate with one or more HA-binding protein(s) and/or polypeptide(s) conjugated to one or more therapeutic agent(s).

10   The critical distinction between the conjugates of the present invention from the subject matter set forth in the Turley PCT application PCT/CA98/00448 mentioned above is that the conjugates of the present invention are not and should not be interposed in any manner with exogenous hyaluronan. The inclusion of hyaluronan in the conjugates of
15   the present invention would dramatically alter their targeting profile thereby obliterating their ability to target to the tissues requiring the desired treatment.

       For example, exogenously administered hyaluronan is entrapped in metabolic organs such as the liver and the interposing of exogenous
20   hyaluronan to the conjugates of the present invention would facilitate the degradation of the conjugate and eliminate the desired therapeutic outcomes of the conjugates. By contrast, conjugates of the present invention when administered without exogenous hyaluronan would target different tissues that are naturally rich in endogenous HA where the desired
25   therapeutic site of action is required.

       One aspect of the present invention provides conjugated sequence(s) of amino acids each having an amino acid sequence encoding an HA-binding protein linked to an effective amount of one or more therapeutic agent(s). Non-limiting examples of such HA-binding protein include
30   receptor for hyaluronan-mediated motility (RHAMM) or a variant or fragment thereof capable of binding HA and anti-HA antibodies.

       A second aspect of the present invention provides conjugated polypeptides each having an amino acid sequence comprising a peptide having HA-binding affinity; linked to an effective amount of therapeutic
35   agent(s). Non-limiting examples of such therapeutic agents include CSF,

EPO, hGH, interferons, PTH and ILs.

Another aspect of the present invention provides isolated and purified nucleic acid sequences encoding an HA-binding peptide in sequence with a therapeutic agent, with or without the presence of an intervening amino acid sequence.

A further aspect of the present invention provides a method for preparing the conjugate molecules of the present invention.

In one embodiment, the preparation of the conjugate molecule comprises the following steps: (i) inserting an appropriate nucleotide sequence (encoding a HA binding protein or peptide) directly or indirectly linked to a nucleotide sequence (encoding a therapeutic protein) into a suitable vector; (ii) expressing the vector in an acceptable host; and (iii) purifying said conjugate molecule from said host or expression medium. In a further embodiment, the preparation of the conjugate molecule comprises the acquisition of the HA binding protein or peptide and therapeutic agent, and the linkage of these domains by one of various chemical and enzymatic methods known to persons skilled in the art.

Also provided is a process for preparing a pharmaceutical for treating an animal in need of treatment comprising the steps of preparing a purified conjugate of the present invention and suspending the conjugate molecule in a pharmaceutically acceptable carrier, diluent or excipient.

A further aspect of the present invention provides a pharmaceutical composition comprising an HA binding protein or peptide directly or indirectly conjugated to a therapeutic agent suspended in a pharmaceutically acceptable carrier, diluent, or excipient.

In yet another aspect, the present invention provides a method for altering *in vivo* the distribution of a therapeutic agent. According to one embodiment the distribution isaltered by conjugating the therapeutic agent to one or more HA-binding protein(s) or peptide(s), administering the conjugate to the animal where the conjugate molecule will distribute primarily in tissues and organs containing high levels of endogenous HA. The present invention also provides a method of treating a mammal with a disorder where the diseased tissue/organ contains high levels of HA by administering the conjugate molecules of the invention to said mammal.

Other features and advantages of the present invention will become

apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit

5   and scope of the invention will become apparent to those skilled in the art from this detailed description.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors found that conjugated therapeutic agents having therapeutic properties when conjugated to a sequence of amino

10   acids which bind HA, surprisingly, have an enhanced ability to target certain diseased tissues and organs enriched in HA. As discussed above, this allows for the use, where appropriate, of lower, safer, dosages of these conjugates as compared to the conventional dosage requirements for the unconjugated corresponding therapeutic agent.

15   The term "HA-binding Protein" as used herein means any polypeptide or protein that can bind hyaluronic acid (or HA). Preferred embodiments of the HA-binding proteins are isolated natural receptors that can bind HA (RHAMM) or antibodies raised against or specifically binds to HA (e.g. Formula I, II, or III). The term HA-binding polypeptide or protein

20 · also includes fragments, analogs and derivatives of RHAMM or anti-HA antibodies, which maintain the ability to bind HA.

The term "HA-binding peptide" as used herein means any peptide that can bind hyaluronic acid (or HA). Preferred embodiments of the HA-binding peptides are of the Formula I, II, or III as defined herein. The term

25   HA-binding peptide includes fragments, analogs and derivatives of the peptides, which maintain the ability to bind HA. Collectively, the HA binding peptides defined herein are referred to as RHAMM peptides. Preferably, RHAMM peptides consist of amino acids in the levorotatory (L) form, which corresponds to the configuration of amino acids in nature.

30   The term "analog" includes any peptide having an amino acid residue sequence substantially identical to the sequence of the HA binding peptides shown in Formula I, II, or III shown herein in which one or more residues have been conservatively substituted with a functionally similar residue and which displays the ability to mimic a HA-binding peptide. Examples of

35   conservative substitutions include the substitution of one non-polar

(hydrophobic) residue such as alanine, isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue

5 such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

The phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue provided that such polypeptide displays the requisite activity.

10 The term "directly" in the context of protein or nucleotide conjugation refers to the linkage of one amino acid or nucleic acid sequence to another without any additional intervening amino acids, nucleotides, or other molecules.

The term "animal" as used herein includes all members of the animal

15 kingdom, including humans. Preferably, the animal to be treated is a human.

The term "indirectly conjugated" refers to the linkage of two amino acid or nucleic acid sequences by the insertion of amino acid residue(s), nucleic acid(s), or other molecule(s) such as a chemical polymer between the

20 two sequences to be linked.

The term "derivative" as used herein refers to a peptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form amine

25 hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of

30 histidine may be derivatized to form N-im-benzylhistidine. Also included as derivatives are those peptides that contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For example: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine;

35 homoserine may be substituted for serine; and ornithine may be substituted

for lysine. HA-binding peptides as provided herein also include any peptide having one or more additions and/or deletions or residues relative to the sequence of a polypeptide whose sequence is shown herein, so long as the requisite activity is maintained or increased.

5   The term "fragment" refers to any subject peptide having an amino acid residue sequence shorter than that of a peptide whose amino acid residue sequence is shown or is being referred to.

Conjugated Proteins

       The present invention provides conjugated proteins or polypeptides
10   each having one or more HA-binding protein(s) or HA-binding peptide(s) linked directly or indirectly to one or more therapeutic agent(s). For greater certainty, one therapeutic agent may be conjugated directly or indirectly to one or more HA-binding protein(s) and/or HA-binding peptide(s), while similarly, one or more therapeutic agent(s) may be conjugated directly or
15   indirectly to an HA-binding protein and/or HA-binding peptide. Without limiting the above, the present inventors also contemplate conjugation of more than one of the same or different therapeutic agents to more than one of the same or different HA-binding proteins and/or HA-binding peptides to form a polymeric scaffold of therapeutic agents, HA-binding proteins
20   and/or HA-binding peptides.

       The selection of therapeutic agents, HA-binding proteins, and HA-binding peptides for conjugation and the stoichiometric ratios of each component to be used would depend on the physicochemical characteristics of the therapeutic agent(s) as well as the pharmacodynamic and
25   pharmacokinetic requirements for the conjugate molecule on a case-by-case basis. In one embodiment, a therapeutic agent is linked directly or indirectly to an HA-binding protein which is a polypeptide comprising an amino acid sequence provided herein as SEQ ID NO. 1, SEQ ID NO. 2 or SEQ ID NO. 3, or a fragment, analog or derivative thereof which maintains the ability to
30   bind HA. In another embodiment, a therapeutic agent is linked directly or indirectly to an HA-binding protein and may be a polyclonal or monoclonal antibody with binding affinity for HA.

       In the event that the therapeutic agent is a polypeptide, the RHAMM-therapeutic protein conjugate may be prepared preferably using
35   conventional recombinant DNA methodology. Alternatively, they may be

produced separately as two sequences and subsequently conjugated by conventional chemical and enzymatic techniques well known to those skilled in the art of coupling of amino acids and peptides.

A second aspect of the present invention is concerned with
5 conjugated polypeptides each comprising an HA-binding peptide sequence linked directly or indirectly to a therapeutic agent. The HA-binding peptide or the entire HA-binding peptide therapeutic protein conjugate may be prepared by chemical synthesis using techniques well known to those skilled in the art of chemistry of proteins, such as solid phase synthesis
10 (Merrifield, 1964) or synthesis in homogenous solution (Houbenweyl, 1987).

In an embodiment, the HA-binding peptide has an amino acid sequence comprising a structure of B^1 - A_n - B^2 wherein B^1 and B^2 are the same or different basic amino acid residues and An is an amino acid sequence containing seven or eight amino acid resides which are the same
15 or different and are neutral or basic amino acids. Preferably, the HA-binding peptide comprises an amino acid sequence selected from the group consisting of KQKKHXXK, KIKHXXKUK, KLRsQLXKIR and KLRSQLXKRK wherein each X is independently selected from V or D (SEQ. ID. NOS.: 7 to 10).

20 In another embodiment, the HA binding peptide that can be conjugated directly or indirectly to a therapeutic protein has an amino acid sequence comprising of the following Formula I:

X_1 - X_2 - X_1 - X_3 - X_4 - X_3 - X_4 - X_3 - X_3 - X_3 - X_5 - X_6 - X_6 - X_6 - X_1
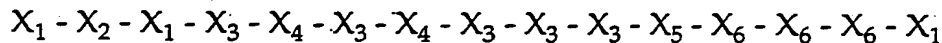
wherein:

25 each X_1 is independently selected from a hydroxy amino acid residue;

each X_2 is independently selected from a sulfur containing amino acid residue;

each X_3 is independently selected from a basic amino acid residue;

each X_4 is independently selected from an imino or aromatic amino acid
30 residue;

each X_5 is independently selected from a dicarboxylic acid amino acid residue; and

each X_6 is independently selected from an aliphatic amino acid residue;

and preferably:

each X_1 is independently selected from threonine or serine;

each X_2 is independently selected from methionine or cysteine;

each X_3 is independently selected from arginine, lysine or histidine;

each X_4 is independently selected from proline, phenylalanine or

5   tryptophan;

each X_5 is independently selected from asparagine or glutamine; and

each X_6 is independently selected from leucine, isoleucine, valine or alanine.

A preferred amino acid sequence of this Formula I is TMTRPHFHKRQLVLS (SEQ. ID. NO.: 11).

10   In a further embodiment, the HA binding peptide that can be conjugated directly or indirectly to a therapeutic protein has an amino acid sequence comprising of the following Formula II:

$Y_1 - Y_1 - Y_2 - Y_2 - Y_1 - Y_3 - Y_1 - Y_3 - Y_3 - Y_1 - Y_3 - Y_1 - Y_2 - Y_3 - Y_3$

wherein:

15   each Y_1 is independently selected from a hydroxy amino acid residue;

each Y_2 is independently selected from a sulfur containing amino acid residue; and

each Y_3 is independently selected from a basic amino acid residue;

and preferably:

20   each Y_1 is independently selected from serine or threonine;

· each Y_2 is independently selected from methionine or cysteine; and

each Y_3 is independently selected from arginine, lysine or histidine.

A preferred amino acid sequence of this Formula II is STMMSRSHKTRSCHH (SEQ. ID. NO.: 12).

25   In another embodiment, the HA binding peptide that can be conjugated directly or indirectly to a therapeutic protein has an amino acid sequence comprising of the following Formula III:

$Z_1 - Z_1 - Z_2 - Z_2 - Z_1 - Z_3 - Z_1 - Z_3 - Z_3 - Z_1 - Z_3 - Z_1 - Z_3 - Z_3$

wherein:

30   each Z_1 is independently selected from a hydroxy amino acid residue;

each Z_2 is independently selected from a sulfur containing amino acid residue; and

each Z_3 is independently selected from a basic amino acid residue, and

fragments;

and preferably,

each Z_1 is independently selected from serine or threonine;

each Z_2 is independently selected from methionine or cysteine; and

5   each Z_3 is independently selected from arginine, lysine or histidine,

and fragments, analogs or derivatives of the peptide which bind HA.

A preferred amino acid sequence of this Formula III is STMMSRSHKTRSHH (SEQ.ID.NO.: 13). This SEQ.ID.NO.: 13 may optionally contain a valine residue at the C-terminal and have the following sequence:

10   STMMSRSHKTRSHHV (SEQ. ID. NO.: 14).

As stated above, the present invention concerns RHAMM peptides and their conjugation to a therapeutic protein. More than half of endogenously produced HA is distributed in skin tissue, and approximately a quarter is distributed in skeletal tissue. Accordingly, a pathological

15   condition or disease condition occuring in these tissues will benefit as therapeutic proteins useful in treating such conditions are targetted to these tissues by a conjugate of the present invention. The therapeutic protein may be chosen from the group comprising a colony stimulating factor (G-CSF, GM-CSF or M-CSF), erythropoietin, growth hormone, parathyroid

20   hormone, an interferon, an interleukin, an antibody, an immunoadhesin, or any functional fragment thereof.

The conjugates of the present invention allow the specific targeting of the therapeutic agent to tissues in which there is a high concentration of HA. An additional feature of the conjugate molecules is an increased half-life and

25   potency, resulting in prolonged circulation of the molecule, efficient distribution into the target tissues, and increased bioavailability. Any therapeutic agent, protein or polypeptide that would benefit from direction to tissues with a high concentration of HA can be conjugated to RHAMM peptide under the present invention.

30   An embodiment of the present invention is the conjugation of a therapeutic protein to an HA-binding protein or HA-binding peptide.

A further embodiment of the present invention is the conjugation of a vaccine or antibody to an HA-binding protein or HA-binding peptide. A vaccine may be conjugated to RHAMM to deliver the vaccine directly to the

35   lymph nodes, which is the site of antibody release into the circulation. As B

and T cells mature, antibodies will recognize the vaccine antigen and induce an immune response, conferring protection against the antigen contained in the vaccine. The conjugation of an antibody (for example to a cancer antigen such as Her-2) to RHAMM could be used in such applications as cancer metastasis, as described above. As tumor cells migrate to the lymph nodes, the RHAMM-antibody conjugate will bind to the cells and induce an immune response, resulting in destruction of the migrating cancer cells.

Antibodies having specificity for a cell surface protein may be prepared by conventional methods. A mammal, (e.g. a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the peptide which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g. the hybridoma technique originally developed by Kohler and Milstein (*Nature* 256:495-497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., *Immunol. Today* 4:72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., Monoclonal Antibodies in Cancer Therapy Allen R., Bliss, Inc., pages 77-96 (1985)), and screening of combinatorial antibody libraries (Huse et al., *Science* 246:1275 (1989)). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated.

As will now be discussed, conjugation techniques may include recombinant production of an amino acid sequence which may be translated

into said conjugate molecule, or direct chemical ligation of the peptide and protein,- ligation of peptide and protein through intermediate linking molecules or sequences.

Methods of Preparing Conjugates

5 *Recombinant Techniques*

Isolated and purified conjugates of the present invention may be prepared using conventional recombinant DNA technology. The preparation of the conjugate molecule comprises the insertion of an appropriate nucleotide sequence (encoding a HA binding peptide directly or 10 indirectly linked to a nucleotide sequence) into a suitable vector, its expression in an acceptable host, and its purification from said host.

The term "isolated and purified" as used herein refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals 15 when chemically synthesized. An "isolated and purified" nucleic acid is also substantially free of sequences which naturally flank the nucleic acid (*i.e.* sequences located at the 5' and 3' ends of the nucleic acid) from which the nucleic acid is derived. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded.

20 The use of recombinant DNA methods to produce the conjugated proteins or unconjugated polypeptides of the present invention involves the insertion of nucleic acids the present invention into a known manner into an appropriate expression vector that ensures good expression of the protein. One embodiment provides isolated and purified nucleic acids having 25 nucleotide sequences encoding an HA-binding peptides in sequence with a therapeutic protein may be synthesized, with or without the presence of an intervening amino acid sequence. The nucleic acid sequences of HA-binding peptides are essentially as those described in PCT patent application PCT/CA93/00158 published as WO 93/21312, Yang et al. (1993), and 30 European patent publication EP 950,708. The nucleic acid sequences of example therapeutic proteins are essentially as those described in: (i) PCT patent application PCT/US84/02021 published as WO 85/02610 for erythropoietin; (ii) European patent publications EP 43,980 and EP 211,148 for alpha-interferon; (iii) U.S. Patent Nos. 4,966,843 and 5376567 for 35 interferon-beta 1 (NCBI Accession Nos. E00171 and CAA23796); (ii)

European patent publications EP 220,574 and EP 536,520 for interferon-beta 2; (iv) U.S. Patent Nos. 4,342,832 and 4,601,980 for human growth hormone; (v) PCT patent application PCT/US86/01708 published as WO 87/01132 for granulocyte-colony stimulating factor (G-CSF); (vi) U.S. Patent Nos. 5,891,429 and 5,942,221 for granulocyte-macrophage-colony stimulating factor (GM-CSF); (vii) European patent publication EP 473,701 and U.S. Patent No. 5,010,010 for (prepro)parathyroid hormone; (viii) European patent publications EP 165,654 and EP 259,160 for interleukin 1; (ix) European patent publications EP 118,977, EP 088,195 and EP 136,489 for interleukin 2; (x) PCT patent application PCT/US89/02599 published as WO 90/01039 and European patent publications EP 314,705 and EP 275,598 for interleukin 3; (xi) European patent publication EP 302,429 for interleukin 4; (xii) PCT patent application PCT/US89/05421 published as WO 90/06370 and European patent publication EP 316,319 for interleukin 6; (xiii) European patent publication EP 506,574 for interleukin 13; and (xiv) European patent publication EP 870,030 for interleukin 16.

The above nucleic acid sequences may each be obtained by chemical synthesis (joining of nucleotides) using conventional methods or by selective amplification of a coding region, using sets of degenerative primers or probes for selectively amplifying the coding region in a genomic or cDNA library. Appropriate primers may be selected from the nucleic acid sequence of HA-binding protein or peptide or a therapeutic protein. It is also possible to design synthetic oligonucleotide primers from the nucleotide sequences for use in PCR. The desired nucleic acid can then be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., *Biochemistry* 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

Possible expression vectors include but are not limited to cosmids, plasmids; or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is compatible with the host cell used. The expression vectors are "suitable for transformation of a host cell", which means that the expression vectors contain a nucleic acid molecule of the invention and regulatory sequences selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid molecule. "Operatively linked" is intended to mean that the nucleic acid is linked to regulatory sequences in a manner that allows expression of the nucleic acid.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary regulatory sequences for the transcription and translation of the inserted protein-sequence. Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will also be appreciated that the necessary regulatory sequences may be supplied by the native A and B chains and/or its flanking regions.

The recombinant expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs, β-galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an

immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. Transcription of the selectable marker gene is monitored by changes in the concentration of the selectable marker protein such as b-galactosidase, chloramphenicol acetyltransferase, or firefly

5   luciferase. If the selectable marker gene encodes a protein conferring antibiotic resistance such as neomycin resistance transformant cells can be selected with G418. Cells that have incorporated the selectable marker gene will survive, while the other cells die. This makes it possible to visualize and assay for expression of recombinant expression vectors of the invention and

10   in particular to determine the effect of a mutation on expression and phenotype. It will be appreciated that selectable markers can be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes that encode a fusion moiety which provides increased expression of the

15   recombinant protein; increased solubility of the recombinant protein; and aid in the purification of the target recombinant protein by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant protein to allow separation of the recombinant protein from the fusion moiety subsequent to purification of

20   the fusion protein. Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the recombinant protein.

25   Recombinant expression vectors can be introduced into host cells to produce a transformant host cell. The term "transformant host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with",

30   "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector) into a cell by one of many possible techniques known in the art. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells via conventional

35   techniques such as calcium phosphate or calcium chloride co-precipitation,

DEAE-dextran mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and

5   other laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. For example, the proteins of the invention may be expressed in bacterial cells such as E. coli, insect cells (using baculovirus), yeast cells or mammalian cells. Other suitable host cells can be found in

10   Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1991).

More particularly, bacterial host cells suitable for carrying out the present invention include E. coli, B. subtilis, Salmonella typhimurium, and various species within the genus' Pseudomonas, Streptomyces, and

15   Staphylococcus, as well as many other bacterial species well known to one of ordinary skill in the art. Suitable bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the b-lactamase (penicillinase) and lactose promoter

20   system (see Chang et al., Nature 275:615 (1978)), the trp promoter (Nichols and Yanofsky, Meth in Enzymology 101:155, 1983) and the tac promoter (Russell et al., Gene 20: 231, 1982). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Suitable expression vectors include but are not

25   limited to bacteriophages such as lambda derivatives or plasmids such as pBR322 (see Bolivar et al., Gene 2:9S, (1977)), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (see Messing, Meth in Enzymology 101:20-77, 1983 and Vieira and Messing, Gene 19:259-268 (1982)), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La Jolla, Calif.). Typical fusion

30   expression vectors which may be used are discussed above, e.g. pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ). Examples of inducible non-fusion expression vectors include pTrc (Amann et al., Gene 69:301-315 (1988)) and pET 11d (Studier et al., Gene Expression Technology: Methods in

35   Enzymology 185, Academic Press, San Diego, California, 60-89 (1990)).

Yeast and fungi host cells suitable for carrying out the present invention include, but are not limited to Saccharomyces cerevisae, the genera Pichia or Kluyveromyces and various species of the genus Aspergillus. Examples of vectors for expression in yeast S. cerivisae include
5   pYepSec1 (Baldari. et al., *Embo J.* 6:229-234 (1987)), pMFa (Kurjan and Herskowitz, *Cell* 30:933-943 (1982)), pJRY88 (Schultz et al., *Gene* 54:113-123 (1987)), and pYES2 (Invitrogen Corporation, San Diego, CA). Protocols for the transformation of yeast and fungi are well known to those of ordinary skill in the art.(see Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929 (1978);
10   Itoh et al., *J. Bacteriology* 153:163 (1983), and Cullen et al. (*Bio/Technology* 5:369 (1987)).

Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g. ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No.
15   CCL 2), 293 (ATCC No. 1573) and NS-1 cells. Suitable expression vectors for directing expression in mammalian cells generally include a promoter (e.g., derived from viral material such as polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40), as well as other transcriptional and translational control sequences. Examples of mammalian expression vectors
20   include pCDM8 (Seed, B., *Nature* 329:840 (1987)) and pMT2PC (Kaufman et al., *EMBO J.* 6:187-195 (1987)).

Given the teachings provided herein, promoters, terminators, and methods for introducing expression vectors of an appropriate type into plant, avian, and insect cells may also be readily accomplished. For example,
25   within one embodiment, the proteins of the invention may be expressed from plant cells (see Sinkar et al., *J. Biosci* (Bangalore) 11:47-58 (1987), which reviews the use of Agrobacterium rhizogenes vectors; see also Zambryski et al., Genetic Engineering, Principles and Methods, Hollaender and Setlow (eds.), Vol. VI, pp. 253-278, Plenum Press, New York (1984), which describes
30   the use of expression vectors for plant cells, including, among others, pAS2022, pAS2023, and pAS2034).

Insect cells suitable for carrying out the present invention include cells and cell lines from Bombyx or Spodotera species. Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include
35   the pAc series (Smith et al., *Mol. Cell Biol.* 3:2156-2165 (1983)) and the pVL

series (Lucklow, V.A., and Summers, M.D., *Virology* 170:31-39 (1989)). Some baculovirus-insect cell expression systems suitable for expression of the recombinant proteins of the invention are described in PCT/US/02442.

Alternatively, the proteins of the invention may also be expressed in non-human transgenic animals such as, rats, rabbits, sheep and pigs (see Hammer et al. (*Nature* 315:680-683 (1985)), Palmiter et al. (*Science* 222:809-814 (1983)), Brinster et al. (*Proc. Natl. Acad. Sci. USA* 82:4438-4442 (1985)), Palmiter and Brinster (*Cell* 41:343-345 (1985)) and U.S. Patent No.4,736,866).

Chemical and Other Synthetic Techniques

The preparation of the conjugate molecules of the present invention may also be achieved through linkage of the HA binding peptide and therapeutic protein by one of various chemical and enzymatic methods known to persons skilled in the art. Many large proteins, including bovine apocytochrome c, human endorphin analogs, beta-lipotropin, human pancreatic growth hormone-releasing factor, inhibin-92, ribonuclease A, bovine pancreatic trypsin inhibitor can be synthesized using various methods (Blake, 1986; Blake and Li, 1981, 1983; Blake et al., 1984, 1986; Cheng and Yamashiro, 1991; Jackson et al., 1994; Lu et al., 1998; Muir et al., 1994; Yamashiro and Li, 1988). Technologies relevant to the present invention include, but are not limited to: engineered protease-mediated ligation; coupling of minimally protected peptides in aqueous solution; backbone-engineered chemical ligation; native chemical ligation; and recombinant DNA technology. These examples are now further described.

Serine proteases have been used to conjugate synthetic, recombinant, or natural proteins and/or peptides with specific terminal sequences. Although the terminal sequence requirements for protease-mediated ligation are not absolute, enzymatic hydrolysis is thermodynamically favored over ligation. Therefore, reaction conditions (pH, temperature, and solvent concentration) may be altered to favor ligation, often at the expense of enzyme stability and substrate solubility (Homandberg, 1978, Wong, 1988). Recent adaptations to this technique have involved activation of the C-terminus of the N-terminal peptide to overcome the thermodynamic barrier. Such techniques are known to persons skilled in the art.

Various mutants of subtilisin have shown an increased ratio of ligase

activity to hydrolysis activity, (Nakatsuka, 1987, Wu and Hilvert, 1989, Abrahmsen, 1991). Specifically, the mutant in which serine 221 is converted to cysteine and proline 225 is converted to alanine has been shown to increase the aminolysis to hydrolysis ratio of tetrapeptide esters and reduce

5   steric crowding at the active site (Jackson et al., 1994). The conjugation procedure can be carried out site specifically in aqueous solution with high yield (Chang et al., 1994). This enzyme has been used in the construction of large proteins, including Ribonuclease A and semisynthesis of various other proteins (reviewed by Braisted et al., 1997).

10   Coupling of minimally protected peptides in aqueous solution will result in ligation of the C-terminus of a peptide to the N-terminus of a protein. In this method, the peptide is synthesized with a thiocarboxyl group (eg. thioglycine) at its C-terminus, while its amino groups and N-terminus are protected with citraconic anhydride. The C-terminal

15   thiocarboxyl group is then activated by the addition of silver nitrate in the presence of N-hydroxysuccinimide, and the peptide is allowed to react with the protein at a pH of 6. Although these conditions favor peptide bond formation at the a-amino group (N-terminus) of the protein, the final product must be purified, as coupling of the peptide to non-terminal amino

20   groups (lysine residues) of the protein may occur. The citraconyl groups may be removed from the conjugate by weak-acid hydrolysis (Dixon and Perham, 1968).

Backbone-engineered chemical ligation results in the conjugation of two peptides or proteins through the creation of a thioester or thioether

25   linkage (Lindner and Robey, 1987; Robey and Fields, 1989). Thioester products are stable below a pH of 6, but are subject to hydrolysis at a pH greater than 7, rendering them inactive at physiological pH and therefore not useful in the scope of the present invention. Reacting a free thiol group on one peptide/protein with a bromoacetyl or iodoacetyl group on another

30   will form a thioether linkage, which is stable at physiological and basic pH (Schnolzer and Kent, 1992). Specifically, the conjugation product of the present invention can be formed by ligating the bromoacetylated or iodoacetylated N-terminus of a protein to a modified RHAMM peptide. The RHAMM peptide is modified to contain a C-terminal Cysteine amide. When

35   the modified peptide and protein are allowed to react, the thiol group on the

peptide attacks the methylene carbon on the acetyl group of the protein, which releases the iodo (bromo) leaving group and results in a thioether linkage between the peptide and protein (Lloyd-Williams, 1997)

Heterobifunctional cross-linkers may be used to conjugate
5   peptides/proteins where one molecule contains a free amine and the second molecule contains (or is modified to contain) a free thiol group and one such cross-linker is sulfo-GMBS (N-_-maleimidobutyryloxysulfo-succinimide ester) (Hermanson, 1996). A method for introducing a protected thiol into amine containing compounds uses a SAMA (S-acetylthioglycolic acid) group
10   (Hermanson, 1996; Duncan, 1983). In the present method, the amine at the N-terminal of a protein is targeted to react with the sulfo-GMBS cross-linker through appropriate use of pH. The linkage between the protein and the cross-linker is a peptide bond and the remainder of the cross-linker contains a thiol reactive maleimide group. A RHAMM peptide is synthesized to
15   contain the SAMA grouping at the N-terminal (*through the use of a pentafluorophenol ester reactive intermediate during peptide synthesis*) which can then be converted into a compound containing a free thiol in a reaction with hydroxylamine just prior to any conjugation experiments. The thus created RHAMM peptide (with reactive thiol) is combined with the modified protein
20   (containing the thiol reactive maleimide group) to form the RHAMM-protein conjugate."

Native chemical ligation involves the ligation of two unprotected peptides or proteins by formation of a thioester, followed by creation of an amide bond between the two sequences. For the purpose of the present
25   invention, this involves ligation of the peptide C-terminus to the N-terminus of the protein. The process requires that the peptide has a C-terminal thioester group, and the protein must have an N-terminal cysteine residue. The reaction takes place at a pH of 6 in phosphate buffer with 6M quanidine. The free thiol group in the cysteine residue will attack the α-carbon of the
30   thioester, resulting in an unstable intermediate that spontaneously rearranges to give an amide bond between the two sequences (Dawson, 1994; Lloyd-Williams et al., 1997).

A linkage sequence of amino acids may be added directly (during direct protein sequencing) or coded by insertion of adjacent nucleic acids
35   (during recombinant synthesis) to the amino or carboxyl terminus of the

peptide and protein. Alternatively, a non-peptide sequence may serve as the linkage between the peptide and protein. The purpose of the linkage molecule or sequence is to form a physical separation between the peptide and the protein in order to prevent steric hindrance between; increase

5   flexibility of the bond between; facilitate attachment and detachment of; and prolong the half-life of; the peptide and protein. This technique is known to persons skilled in the art, and often yields more active complexes (Hermanson, 1996). Such linkage may be chosen from, but is not limited to, the group comprising: the constant domain of an antibody; the hinge region

10 of an antibody; an amino acid sequence which permits enzymatic ligation of the two peptides (for example, when cysteine is required at the N-terminus of the protein for enzymatic ligation; polyethylene glycol; an amino acid sequence of sufficient length; and a chemical linkage and attached group.

Therapeutic Formulations of the Present Invention

15   The conjugate proteins of the invention may be formulated into pharmaceutical compositions for adminstration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration in vivo" is meant a form of the substance to be administered in which any toxic effects are outweighed by

20 the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a

25 therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, the route of drug administration and the ability of drug to elicit a desired response in the individual.

        The active substance may be administered in a convenient manner

30 such as injection (subcutaneous, intravenous, intramuscular, etc.), oral administration, inhalation, transdermal administration (such as topical cream or ointment, etc.), or suppository applications. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural

conditions which may inactivate the compound. The nature of said coated preparations may range from conventional enteric-coated formulations (Wilding, I.R. et al., *Pharmacol. Ther.* 62: 97-124 (1994)) to liposomal or microsphere formulations consisting unilamellar or multilamellar vesicles
5 containing the active substance for oral, parenteral or pulmonary administration. Prepararation of liposomes or microspheres have been described (Constantinides, P.P., *Pharm. Res.* 12: 1561-1572 (1995); Jones, M.N., *Adv. Colloid Interface Sci.* 54: 93-128 (1995); Cohen, S. et al., *Int. J. Technol. Assess. Health Care* 10: 121-130 (1994)); Kinnunen, P.K. et al., *Chem.*
10 *Phys. Lipids* 73: 181-207 (1994). Extensive research to improve their physicochemical properties and pharmacokinetics have also been documented (Hnatyszyn, H.J. et al., *PDA J. Pharm. Sci. Technol.* 48: 247-254 (1994); Patel, N.M., *Crit. Rev. Ther. Drug Carrier Syst.* 9: 39-90 (1992)).

The compositions described herein can be prepared by *per se* known
15 methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences,
20 Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

25 The pharmaceutical compositions of the present invention may be used in methods for treating animals, including humans, with a disorder that can be treated by the corresponding therapeutic protein. Said compositions and preparations may contain a conjugate protein, either alone, or in combination with other active substances.

30 In particular, compositions for parenteral administration can be prepared as solutions of the conjugate protein or as powders of the conjugate protein to be mixed with one or more pharmaceutically acceptable excipients or diluents, suitable for the aforesaid uses and with an osmolarity which is compatible with the physiological fluids. For example,
35 as described herein, said preparation may be formulated with a wetting

agent and/or stabilized by addition of a stabilizer. When administering the compositions/preparations of the invention by injection, the administration may be by continuous infusion or by single or multiple injections. In an embodiment of the invention, forms for intravenous injection or infusion

5 are selected to maximize drug availability, reduce dosage, and to elicit faster pharmacodynamic action.

The compositions and preparations of the invention are intended to provide to the recipient subjects an amount of a conjugated therapeutic protein sufficient to treat the symptoms of or prevent a disease or disorder

10 treatable by the native therapeutic protein. Dosages of a conjugated protein to be administered depend largely on individual needs, on the desired effect in and duration a particular therapeutic indication, and on the chosen route of drug administration.

Administration of a therapeutically active amount of the

15 pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, the route of drug administration and the

20 ability of drug to elicit a desired response in the individual. Determination of a a therapeutically active amount of the pharmaceutical compositions of the present invention also depends on the stoichiometric ratio for the number of HA-binding peptide or protein(s) conjugated to the number of therapeutic agent. In addition, the molecular size of the HA-binding protein

25 will also affect dosage on a gram per body weight or body surface area basis.

For example, under the assumption that: (i) the HA-binding domain is a peptide with an amino acid sequence as set forth as SEQ ID NO.: 13; (ii) the therapeutic agent is granulocyte-macrophage colony stinulating factor

30 (GM-CSF) or granulocyte colony stinulating factor (G-CSF); and (iii) the stoichiometric ratio between HA-binding peptide and therapeutic agent is 1:1, the recommended daily parenteral doses of the HA-bind-peptide-GM-CSF and HA-bind-peptide-G-CSF conjugates would range from about 0.01μg/kg/day to about 30μg/kg/day and preferably between 0.5

35 μg/kg/day to 10 μg/kg/day given by intravenous infusion or

subcutaneous injection.

Similarly, if: (i) the HA-binding domain is a peptide with an amino acid sequence as set forth as SEQ ID NO.: 13; (ii) the therapeutic agent is erythropoietin; and (iii) the stoichiometric ratio between HA-binding peptide and therapeutic agent is 1:1, the recommended daily parenteral dose of HA-bind-peptide-erythropoietin conjugate would range from about 1 U/kg/day to 1000 U/kg/day and preferably between 3 U/kg/day to 300 U/kg/day given by intravenous or subcutaneous injection.

Further, if: (i) the HA-binding domain is a peptide with an amino acid sequence as set forth as SEQ ID NO.: 13; (ii) the therapeutic agent is human growth hormone; and (iii) the stoichiometric ratio between HA-binding peptide and therapeutic agent is 1:1, the recommended daily parenteral dose of HA-bind-peptide-human growth hormone conjugate would range from about 0.001 IU/kg/day to 10 IU/kg/day and preferably between 0.05 IU/kg/day to 0.2 IU/kg/day given by subcutaneous or intramuscular injection.

Yet further, if: (i) the HA-binding domain is a peptide with an amino acid sequence as set forth as SEQ ID NO.: 13; (ii) the therapeutic agent is an alpha-interferon; and (iii) the stoichiometric ratio between HA-binding peptide and therapeutic agent is 1:1, the recommended daily parenteral dose of HA-bind-peptide-alpha-interferon conjugate would range from about 0.001 MIU/kg/day to 1 MIU/kg/day and preferably between 0.005 IU/kg/day to 0.5 MIU/kg/day given by subcutaneous or intramuscular injection.

The term "antibody" as used herein is intended to include fragments thereof which also specifically react with a cell surface component. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above. For example, F(ab')2 fragments can be generated by treating antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

Chimeric antibody derivatives, i.e. antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an

antibody of a mouse, rat, or other species, with human constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes a cell surface antigen (See, for example, Morrison et al., *Proc. Natl Acad. Sci. U.S.A.* 81:6851 (1985);

5   Takeda et al., *Nature* 314:452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., E.P. Patent No. 171,496; European Patent No. 173,494, United Kingdom Patent No. GB 2177096B). It is expected that chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric

10   antibody.

Monoclonal or chimeric antibodies specifically reactive against cell surface components can be further humanized by producing human constant region chimeras, in which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of

15   human origin and only the hypervariable regions are of non-human origin. Such immunoglobulin molecules may be made by techniques known in the art, (e.g. Teng et al., *Proc. Natl. Acad. Sci. U.S.A.*, 80:7308-7312 (1983); Kozbor et al., *Immunology Today* 4:7279 (1983); Olsson et al., *Meth. Enzymol.*, 92:3-16 (1982), and PCT Publication WO92/06193 or EP 239,400). Humanized

20   antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

Specific antibodies, or antibody fragments, reactive against cell surface components may also be generated by screening expression libraries encoding immunoglobulin genes, or portions thereof, expressed in

25   bacteria with cell surface components. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries (See for example Ward et al., *Nature* 341:544-546 (1989); Huse et al., *Science* 246:1275-1281 (1989); and McCafferty et al., *Nature* 348:552-554 (1990)). Alternatively, a SCID-hu mouse, for example the model

30   developed by Genpharm, can be used to produce antibodies, or fragments thereof.

Dosage regime may be adjusted to provide the optimum therapeutic response. For example, the daily doses may be combined into a less frequent dosage regimen (e.g. to three-times weekly or t.i.w.), several

35   divided doses may be administered on a given day (e.g. three-times per day

or t.i.d.) or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following specific embodiments are therefore to be construed as merely illustrative and not limiting of the remainder of the disclosure in any way whatsoever. The following examples serve as illustrations of the invention only, and are specifically directed to conjugation techniques and therapeutic proteins for use in the present invention.

EXAMPLE 1

Production of RHAMM-EPO direct conjugate using backbone engineered thioether linkage

Erythropoietin would benefit substantially from the distribution of HA in bone marrow, as targeted by RHAMM peptides. Recombinant erythropoietin is currently produced in the glycosylated form, as the naked form is readily degraded and, therefore, therapeutically ineffective. Therefore, conjugation of non-glycosylated EPO to a RHAMM peptide will increase the half-life and enhance the tissue targeting of EPO and direct the conjugate to tissues with a high concentration of HA. It has been reported that approximately 25% of HA is found in bone, which is an optimal target for EPO, and as such, in addition to increasing half-life, conjugation to RHAMM may enhance target specificity and the therapeutic action of erythropoietin for example in the treatment of anemia.

Preparation of recombinant EPO

The complete coding DNA sequence of human erythropoietin was first disclosed in US patent application Ser. No. 655.841, filed September 28, 1984, which was later issued as US Patent Number 4,703,008 [GenBank Accession No. AH009003 or E00630]. Recombinant EPO can be produced by methods known to persons skilled in the art, or purchased from existing commercial sources. For this method of coupling, the protein does not require modification or engineering prior to the conjugation step.

A sulfhydryl group must be added to the nitrogen of the lysine

residue of EPO for attachment of the RHAMM peptide. This may be accomplished by dissolving EPO in 50 mM phosphate buffer and 150 mM NaCl at a pH of 8. 2-iminothiolane (2-IT) is added at a 10-fold molar excess, followed by incubation for one hour at room temperature (Hermanson, 1996; Traut et al., 1973). Following thiolation, the protein is purified by SEC and lyophilized. The preparation of recombinant EPO for thioether linkage conjugation to RHAMM is illustrated structurally below, where H2N- and –OH represent the N and C terminals of EPO, respectively.

$$H_2N^{\alpha}\text{-EPO-(LysN}^{\varepsilon}H_2)\text{-OH}$$

STEP 1:

    1) pH8
    2) 2-IT

$$H_2N^{\alpha}\text{-EPO[LysN}^{\varepsilon}HC(=NH_2^+)\text{-CH}_2CH_2CH_2\text{-SH]-OH}$$

Preparation of RHAMM peptide

For the purpose of illustration in this example, the HA-binding domain has an amino acid sequence as defined in SEQ. I.D. 13. It should be appreciated by those skilled in the art that other amino acid sequences representing other HA-binding peptides or polypeptides as contemplated hereunder shall constitute equivalent substitutions without departure from the principles of the present invention.

The RHAMM peptide must be engineered with an N-terminal bromoacetyl group, which will later be ligated to the thioloated lysine residues. To this end, the RHAMM peptide is coupled to a hydroxymethyl resin if a free acid is desired, or to a benzhydrylamine resin for a C-terminal amide. RHAMM is then synthesized as per normal Boc or Fmoc solid phase peptide synthesis (SPPS) methods. Following complete synthesis of the RHAMM peptide, the peptide is deprotected and coupled with bromoacetic anhydride (Robey, 1994; Robey and Fields, 1989). Finally, SEC and/or RP-HPLC methods are used to purify the peptide. The process is illustrated structurally below.

$$\text{Boc-aa} + \text{HOCH}_2\text{-Resin \{C terminal COOH\}}$$

OR   Boc-aa + H$_2$N-Resin {C terminal CONH$_2$}

$\downarrow$

STEP 2:

1) Boc synthesis of RHAMM peptide

2) deprotect

3) Bromoacetic acid anhydride

5   Br-CH$_2$CON$^\alpha$H-RHAMM-OH

Coupling of the RHAMM peptide and EPO

Thiolated EPO is dissolved in 0.1 M NaHCO$_3$. The solid bromoacetylated RHAMM peptide is added to the solution, the pH adjusted to 8, and the solution is allowed to incubate at room temperature for 3

10   hours (Robey and Fields, 1989, Kolodny and Robey, 1990, Muir et al., 1994). Purification of the conjugate is by SEC and/or RP-HPLC. The structural representation of the conjugation reaction is illustrated below.
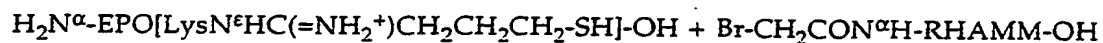
H$_2$N$^\alpha$-EPO[LysN$^\epsilon$HC(=NH$_2$$^+$)CH$_2CH_2CH_2$-SH]-OH + Br-CH$_2CON^\alpha$H-RHAMM-OH

STEP 3: $\downarrow$ 1) pH 8

15

2) incubate at room temperature 3h

3) purify by SEC and/or RP-HPLC

H$_2$N$^\alpha$-EPO[LysN$^\epsilon$HC(=NH$_2$)CH$_2$CH$_2$CH$_2$-S-CH$_2$CONH-RHAMM-OH]-OH

Characterization

The level of conjugation can be assessed by determining the mass of

20   the conjugate, and a tryptic digestion map will determine the point of substitution.

EXAMPLE 2

Conjugation of RHAMM peptide and hGH by the coupling of minimally protected peptides

Human growth hormone (hGH) binds to cell-surface receptors throughout the body to stimulate organ and tissue growth, and of specific relevance to the present invention is the anabolic effect of hGH on bone formation in the epiphyses of long bones. hGH is therefore beneficial in 5 growth insufficiencies and wasting disorders; however, excessive use of hGH has been associated with onset of diabetes and an increased incidence of osteoporosis and heart disease. The conjugation of hGH to RHAMM peptide is expected to increase distribution of hGH to the bone marrow, allowing a reduction in dosage requirements, and a decreased incidence of 10 insulin-like side effects with respect to glucose metabolism as well as other hGH-induced toxicities.

Preparation of recombinant hGH

The complete coding sequence of human growth hormone is given as GenBank Accession No. E00141, E01424 or A15072. For this method of 15 coupling, the protein does not require modification or engineering prior to the conjugation step. Recombinant hGH may be purchased from existing commercial sources, or produced by methods known to persons skilled in the art.

Preparation of RHAMM peptide

20 For the purpose of illustration in this example, the HA-binding domain has an amino acid sequence as defined in SEQ. I.D. 13. It should be appreciated by those skilled in the art that other amino acid sequences representing other HA-binding peptides or polypeptides as contemplated hereunder shall constitute equivalent substitutions without departure from 25 the principles of the present invention.

The RHAMM peptide and is synthesized with a C-terminal thiocarboxyl moiety, which may be accomplished by standard SPPS methods using the following procedure. Boc-thioglycine is coupled to a carboxyl handle, and then to an aminomethyl resin in the presence of 30 dicyclohexylcarbodiimide (DCC) (Baca, 1995, Blake, 1981, and Yamashiro, 1988). The RHAMM peptide can then be synthesized using standard Boc SPPS protocols and cleaved from the resin with HF to give RHAMM-Gly-thiocarboxyl. Prior to conjugation of the engineered RHAMM peptide with

hGH, the amino groups of RHAMM must be blocked. This is accomplished by dissolving the modified RHAMM peptide in potassium phosphate buffer at pH 7.5, followed by the addition of citraconic anhydride dissolved in dioxane. The modified and blocked RHAMM peptide is then purified by

5   SEC in 50 mM ammonium bicarbonate and lyophilized. The preparation of the RHAMM peptide for silver ion activation of the thiocarboxyl C-terminus is illustrated structurally below.

Boc-Gly-COSH   +   [Handle]-COOH

STEP 1:   ↓

10   Boc-Gly-COS-[Handle]-C-OH

STEP 2:   ↓ DCC + H$_2$N-CH$_2$-Resin

Boc-Gly-COS-[Handle]-CONH-Resin

STEP 3:   ↓   1) RHAMM synthesis by Boc SPPS method
                2) HF

15   H$_2$N$^\alpha$-RHAMM-Gly-SH

STEP 4:   ↓   Citraconic anhydride

cit-N$^\alpha$H-RHAMM(LysN$^\epsilon$cit)-Gly-SH

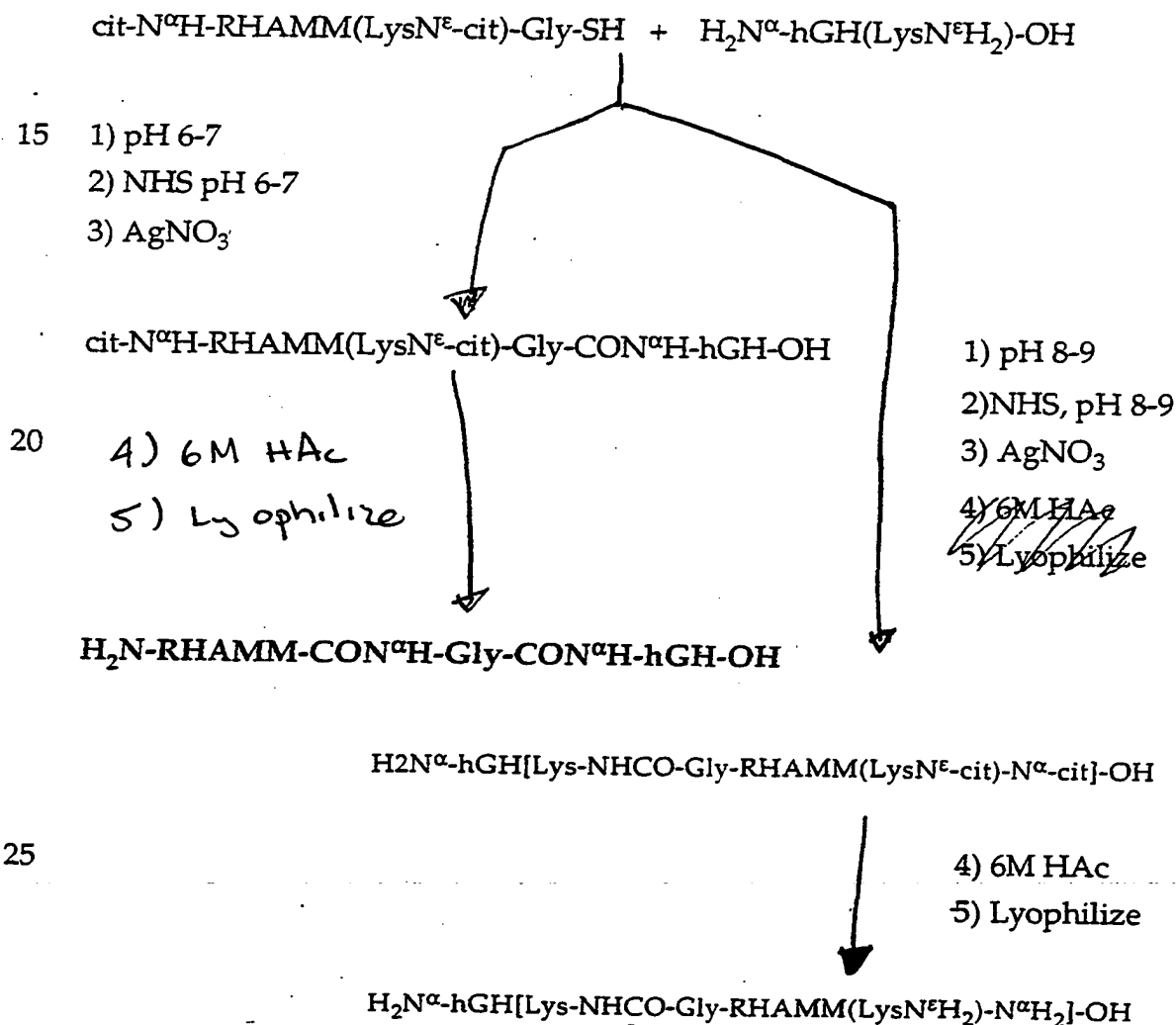*Coupling of RHAMM peptide and hGH*

For the coupling of the RHAMM peptide to the ε amino groups on

20   the lysine residues of the protein, conjugation should take place at a pH of 8-

9. If conjugation between the RHAMM peptide and predominantly the α amino group (N-terminus) of the protein is preferred, conjugation should take place at pH 6-7.

Both peptide and protein are dissolved in water and pH adjusted as
5 desired. This is followed by the addition of N-hydroxysuccinimide at 0°C (also at desired pH). AgNO$_3$ is added, and the solution is incubated at 0°C for 30 minutes and at room temperature for 45 minutes (Blake and Li, 1981, 1983). The protective citraconyl groups are removed by incubation with a large excess of 6M acetic acid for 2.5 hours. The final product is lyophilized
10 and purified by RP-HPLC using water/TFA and acetonitrile/TFA. The product is eluted by buffer exchange into an appropriate buffer using SEC. The coupling reaction between hGH and prepared RHAMM peptide is illustrated structurally below.

cit-N$^\alpha$H-RHAMM(LysN$^\epsilon$-cit)-Gly-SH   +   H$_2$N$^\alpha$-hGH(LysN$^\epsilon$H$_2$)-OH

15   1) pH 6-7
     2) NHS pH 6-7
     3) AgNO$_3$

cit-N$^\alpha$H-RHAMM(LysN$^\epsilon$-cit)-Gly-CON$^\alpha$H-hGH-OH   1) pH 8-9
                                                                          2)NHS, pH 8-9
20   4) 6M HAc   3) AgNO$_3$
     5) Lyophilize   4) 6M HAc
                                                                          5) Lyophilize

H$_2$N-RHAMM-CON$^\alpha$H-Gly-CON$^\alpha$H-hGH-OH

H2N$^\alpha$-hGH[Lys-NHCO-Gly-RHAMM(LysN$^\epsilon$-cit)-N$^\alpha$-cit]-OH

25   4) 6M HAc
                                                                         5) Lyophilize

H$_2$N$^\alpha$-hGH[Lys-NHCO-Gly-RHAMM(LysN$^\epsilon$H$_2$)-N$^\alpha$H$_2$]-OH

Characterization

The level of conjugation can be assessed by determining the mass of the conjugate, and a tryptic digestion map will determine the point of substitution (if not N-terminal).
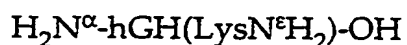
5 **EXAMPLE 3**

Conjugation of RHAMM peptide to hGH by backbone engineered thioether linkage using a bifunctional PEG linkage

Preparation of recombinant hGH

The complete coding sequence of human growth hormone is given as
10 GenBank Accession No. E00141, E01424 or A15072. For this method of coupling, the protein does not require modification or engineering prior to the conjugation step. Recombinant hGH may be purchased from existing commercial sources, or produced by methods known to persons skilled in the art.

15 A sulfhydryl group must be added to the ε nitrogen of the lysine residue of hGH for attachment of the RHAMM peptide. This may be accomplished by dissolving hGH in 50 mM phosphate buffer and 150 mM NaCl at a pH of 8. 2-iminothiolane (2-IT) is added at a 10-fold molar excess, followed by incubation for one hour at room temperature (Hermanson,
20 1996; Traut et al., 1973). Following thiolation, the protein is purified by SEC and lyophilized. The preparation of recombinant hGH for thioether linkage conjugation to RHAMM is illustrated structurally below.

$$H_2N^\alpha\text{-hGH(LysN}^\epsilon H_2)\text{-OH}$$

STEP 1: $\downarrow$ 1) pH8

25   2) 2-IT

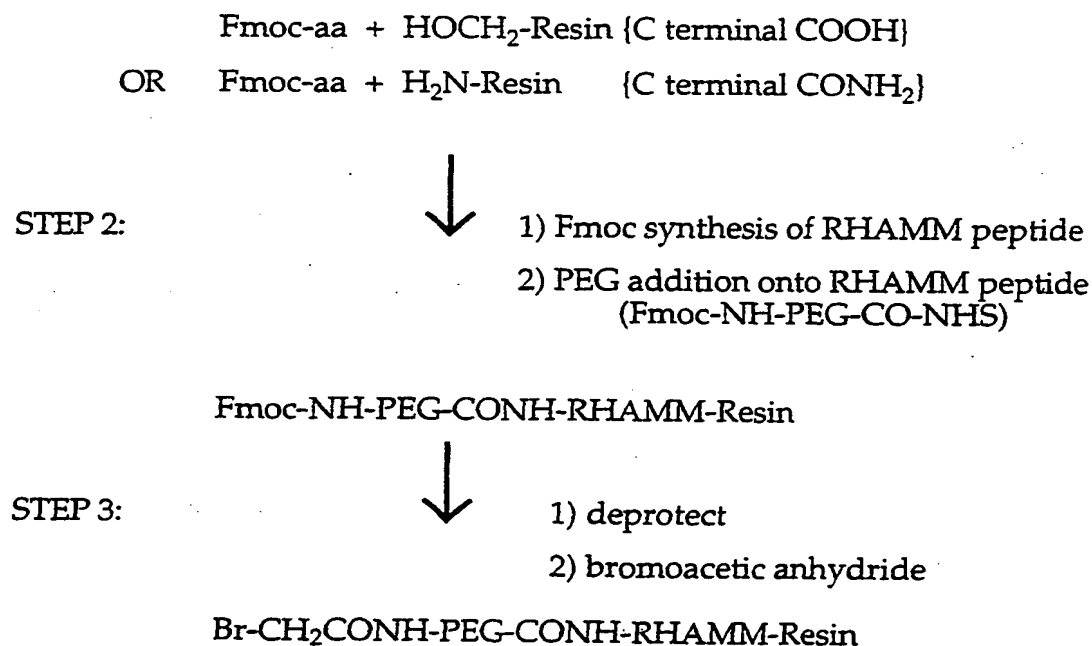$$H_2N^\alpha\text{-hGH[LysN}^\epsilon HC(=NH_2^+)CH_2CH_2CH_2\text{-SH]-OH}$$

Preparation of N-terminal bromoacetylated RHAMM peptide

For the purpose of illustration in this example, the HA-binding

domain has an amino acid sequence as defined in SEQ. I.D. 13. It should be appreciated by those skilled in the art that other amino acid sequences representing other HA-binding peptides or polypeptides as contemplated hereunder shall constitute equivalent substitutions without departure from the principles of the present invention. The RHAMM peptide-PEG conjugate must be engineered with an N-terminal bromoacetyl group, which will later be ligated to the lysine residues on hGH that have been thiolated. To this end, the RHAMM peptide is coupled to a hydroxymethyl resin if a free acid is desired, or to a benzhydrylamine resin for a C-terminal amide. RHAMM is then synthesized as per normal Fmoc SPPS methods (Boc methods are not recommended for this procedure (Lu and Felix, 1993)). Following complete synthesis of the RHAMM peptide, the bifunctional PEG molecule is coupled to the fully synthesized RHAMM peptide as per standard Fmoc SPPS methods. The N-terminal of the RHAMM-PEG conjugate is then deprotected and coupled with bromoacetic anhydride. Finally, SEC and/or RP-HPLC methods are used to purify the peptide. The process is illustrated structurally below.

$$\text{Fmoc-aa} + \text{HOCH}_2\text{-Resin} \quad \{\text{C terminal COOH}\}$$

$$\text{OR} \quad \text{Fmoc-aa} + \text{H}_2\text{N-Resin} \quad \{\text{C terminal CONH}_2\}$$

$$\downarrow$$

STEP 2: 1) Fmoc synthesis of RHAMM peptide

2) PEG addition onto RHAMM peptide (Fmoc-NH-PEG-CO-NHS)

Fmoc-NH-PEG-CONH-RHAMM-Resin

$$\downarrow$$

STEP 3: 1) deprotect

2) bromoacetic anhydride

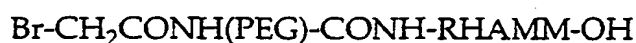Br-CH$_2$CONH-PEG-CONH-RHAMM-Resin

Coupling of RHAMM-PEG to hGH

Thiolated hGH is dissolved in 0.1 M NaHCO$_3$. The bromoacetylated

RHAMM-PEG conjugate is added to the solution, the pH adjusted to 8, and the solution is allowed to incubate at room temperature for 3 hours (Robey and Fields, 1989, Kolodny and Robey, 1990, Muir et al., 1994). Purification of the conjugate is by SEC and/or RP-HPLC. The structural representation of
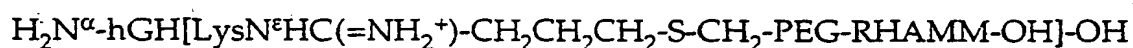
5   the conjugation reaction is illustrated below.

$$H_2N^\alpha\text{-hGH[LysN}^\varepsilon HC(=NH_2^+)CH_2CH_2CH_2\text{-SH]-OH}$$

$$+$$

$$Br\text{-}CH_2CONH(PEG)\text{-}CONH\text{-}RHAMM\text{-}OH$$

$$\downarrow$$

STEP 4:   1) pH 8

10   2) incubate at room temperature 3h
                                 3) purify by SEC and/or RP-HPLC

$$H_2N^\alpha\text{-hGH[LysN}^\varepsilon HC(=NH_2^+)\text{-}CH_2CH_2CH_2\text{-}S\text{-}CH_2\text{-}PEG\text{-}RHAMM\text{-}OH]\text{-}OH$$

A dosage of 0.06-0.1 mg (0.16-0.026 IU) hGH per kg body weight 3 times weekly is recommended for the treatment of growth deficiency

15   associated with growth hormone insufficiency. For the treatment of growth failure in patients with Turner's syndrome, dosage of up to 1 IU/kg/week is recommended (or 24 IU/m2). Adverse effects of hGH treatment include antibody production, intracranial hypertension, and increased incidences of tumor and leukemia. Excessive use of hGH has been associated with onset

20   of diabetes and an increased incidence of osteoporosis and heart disease.

EXAMPLE 4
Conjugation of RHAMM peptide to EPO by coupling of minimally protected peptides by silver ion activation of thiocarboxyl C-terminus using a bifunctional PEG molecule as a linker

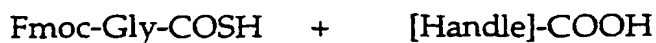25   *Preparation of recombinant EPO*
        The complete coding DNA sequence of human erythropoietin was first disclosed in US patent application Ser. No. 655.841, filed September 28, 1984, which was later issued as US Patent Number 4,703,008 [GenBank Accession No. AH009003 or E00630]. Recombinant EPO can be produced by

methods known to persons skilled in the art, or purchased from existing commercial sources. For this method of coupling, the protein does not require modification or engineering prior to the conjugation step.
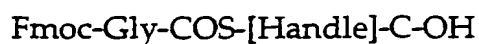
Recommended dose of EPO ranges from 75U/kg to 600U/kg thrice
5 weekly depending on the indication. Adverse effects include allergic reaction and antibody induction, clotting of dialyzer/artificial kidney, hypertension, seizures, headache, arthralgia, tachycardia, nausea, clotted vascular access, shortness of breath, hyperkalemia, and diarrhea.

Preparation of RHAMM-PEG

10 For the purpose of illustration in this example, the HA-binding domain has an amino acid sequence as defined in SEQ. I.D. 13. It should be appreciated by those skilled in the art that other amino acid sequences representing other HA-binding peptides or polypeptides as contemplated hereunder shall constitute equivalent substitutions without departure from
15 the principles of the present invention. The RHAMM peptide and is synthesized with a C-terminal thiocarboxyl moiety, which may be accomplished by incorporating a bifunctional PEG molecule onto the end of RHAMM. The PEG molecule serves both as a thiocarboxyl terminal group, and as a physically extended linkage between RHAMM and EPO. Fmoc-
20 thioglycine is coupled to a carboxyl handle, and then to an aminomethyl resin in the presence of dicyclohexylcarbodiimide (DCC) (Baca, 1995, Blake, 1981, and Yamashiro, 1988). The bifunctional PEG molecule is then coupled to RHAMM as per normal Fmoc SPPS. The PEG-RHAMM molecule is cleaved from the resin with HF to give RHAMM-PEG-Gly-SH. Prior to
25 conjugation of the engineered RHAMM-PEG with EPO, the amino groups of RHAMM must be blocked. This is accomplished by dissolving the RHAMM-PEG conjugate in potassium phosphate buffer at pH 7.5, followed by the addition of citraconic anhydride dissolved in dioxane. The modified and blocked RHAMM-PEG conjugate is then purified by SEC in 50mM
30 ammonium bicarbonate and lyophilized. The preparation of the RHAMM peptide for silver ion activation of the thiocarboxyl C-terminus is illustrated structurally below.

Fmoc-Gly-COSH   +   [Handle]-COOH

STEP 1:   ↓

Fmoc-Gly-COS-[Handle]-C-OH

STEP 2:   ↓   DCC + H2N-CH2-Resin

5   Fmoc-Gly-COS-[Handle]-CONH-Resin

STEP 3:   ↓   1) RHAMM synthesis by Fmoc method
                     2) add bifunctional PEG to RHAMM by Fmoc method
                     3) HF

H_2N^α-RHAMM-CONH-PEG-CONH-Gly-SH

10   STEP 4:   ↓   Citraconic anhydride

cit-N^αH-RHAMM(LysN$^\epsilon$cit)-PEG-Gly-SH

Conjugation of RHAMM-PEG to EPO

For the coupling of the RHAMM-PEG to the ε amino groups on the
lysine residues of the protein, conjugation should take place at a pH of 8-9.
15   If conjugation between RHAMM-PEG and predominantly the α amino
group (N-terminus) of the protein is preferred, conjugation should take
place at pH 6-7.

Both peptide and EPO are dissolved in water and pH adjusted as
desired. This is followed by the addition of N-hydroxysuccinimide at 0°C
20   (also at desired pH). AgNO$_3$ is added, and the solution is incubated at 0°C

for 30 minutes and at room temperature for 45 minutes (Blake and Li, 1981, 1983). The protective citraconyl groups are removed by incubation with a large excess of 6M acetic acid for 2.5 hours. The final product is lyophilized and purified by RP-HPLC using water/TFA and acetonitrile/TFA. The

5   product is eluted by buffer exchange into an appropriate buffer using SEC. The coupling reaction between EPO and prepared RHAMM-PEG is illustrated structurally below.

cit-N$^\alpha$H-RHAMM(LysN$^\varepsilon$-cit)-PEG-Gly-SH + H$_2$N$^\alpha$-EPO(LysN$^\varepsilon$H$_2$)-OH

10   1) pH 6-7
     2) NHS pH 6-7
     3) AgNO$_3$

cit-N$^\alpha$H-RHAMM(LysN$^\varepsilon$-cit)-PEG-Gly-CON$^\alpha$H-EPO(Lys-N$^\varepsilon$H2)-OH

                                              1) pH 8-9
                                              2)NHS, pH 8-9
15   4) 6M Hac   3) AgNO$_3$
     5) Lyophilize

H$_2$N$^\alpha$-RHAMM(LysN$^\varepsilon$H$_2$)-PEG-Gly-CON$^\alpha$H-EPO(Lys-N$^\varepsilon$H2)-OH

H$_2$N$^\alpha$-EPO-[LysNHCO-Gly-PEG-RHAMM(LysN$^\varepsilon$-cit)-N$^\alpha$Hcit]-OH

                                              4) 6M HAc
20   5) Lyophilize

H$_2$N$^\alpha$-EPO-[LysNHCO-Gly-PEG-RHAMM-N$^\alpha$H$_2$]-OH

EXAMPLE 5

Conjugation of RHAMM peptide and GM-CSF by the coupling of minimally
25   ### protected peptides

The colony stimulating factors (G-CSF and GM-CSF) are natural haematopoietic growth factors that regulate the production and functional activity of granulocytes, macrophages, and mature white cells. These white cells form the basis of the body's immune system, which may be

30   compromised by events ranging from chemotherapy or infection to idiopathic neutropenia. These agents are useful in reducing the duration of hospitalization, intensity of hospitalized treatment, and antibiotic usage

among cancer patients, improve overall prognosis, and permit higher, more frequent doses of chemotherapy. Therefore, G-CSF and GM-CSF are useful in the treatment of cancer, and may be used to stimulate proliferation of cell colonies in various tissues. The conjugation of a CSF to RHAMM would exploit the distribution of endogenous HA, allowing RHAMM to direct the CSF to tissues rich in HA, including the lymph nodes and bone marrow. This technology would be useful in the mobilization of HIV, which lies dormant in lymph nodes following antiretroviral treatment. The specific targeting of the CSF to the lymph nodes is expected to stimulate the proliferation of cells harboring the latent virus, allowing the antiretroviral therapy to eradicate the virus. As the majority of hematopoiesis occurs in bone marrow, distribution of the CSF to the bone marrow would stimulate myeloid reconstitution and stem cell mobilization following destruction of immune cells, such as in chemotherapy, neutropenia, and bone marrow or stem cell transplantation.

Another application of a CSF-RHAMM conjugate would be in cancer metastasis, when tumor cells migrate to the lymph nodes. Increased concentrations of HA have been documented in cancer cells as well as in lymph nodes towards which a conjugate of the present invention could target. Such a conjugate would preferably contain a HA-binding domain linked directly or indirectly to tumor necrosis factor (TNF), interleukin (IL), interferon (IFN), and/or other chemotherapeutic agents.

The recommended daily parenteral doses of GM-CSF and G-CSF range from about $1\mu g/kg/day$ to about $20\mu g/kg/day$ preferably for 21 days. The injection is generally administered in 2-hour i.v. infusions, or as subcutaneous injections. At these doses, notable low-grade toxicities include headache, peripheral edema, arthralgia, myalgia, and skin rash. Pericarditis is the major dose-limiting side effect for GM-CSF at high dosages, and is contraindicated in patients with excessive myeloblasts in bone marrow or known hypersensitivity to expression-host proteins.

The recommended dose of G-CSF is $5-10\mu g/kg/day$ for up to 21 days. Major low-grade adverse reactions associated with G-CSF therapy include medullary bone pain, and spontaneously reversible elevations in uric acid, lactate dehydrogenase, and alkaline phosphatase. The only reported dose-limiting side effect of G-CSF therapy is Sweet syndrome

(acute febrile neutrophilic dermatosis).

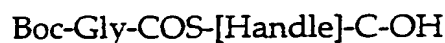*Preparation of recombinant GM-CSF*

The complete coding sequence of human GM-CSF is given as GenBank Accession No. E00951. For this method of coupling, the protein

5   does not require modification or engineering prior to the conjugation step. Recombinant GM-CSF may be purchased from existing commercial sources, or produced by methods known to persons skilled in the art.
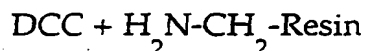
Preparation of RHAMM peptide

For the purpose of illustration in this example, the HA-binding

10   domain has an amino acid sequence as defined in SEQ. I.D. 13. It should be appreciated by those skilled in the art that other amino acid sequences representing other HA-binding peptides or polypeptides as contemplated hereunder shall constitute equivalent substitutions without departure from the principles of the present invention. The RHAMM peptide and is

15   synthesized with a C-terminal thiocarboxyl moiety, which may be accomplished by standard SPPS methods using the following procedure. Boc-thioglycine is coupled to a carboxyl handle, and then to an aminomethyl resin in the presence of dicyclohexylcarbodiimide (DCC) (Baca, 1995, Blake, 1981, and Yamashiro, 1988). The RHAMM peptide can then be synthesized

20   using standard Boc SPPS protocols and cleaved from the resin with HF to give RHAMM-Gly-thiocarboxyl. Prior to conjugation of the engineered RHAMM peptide with hGH, the amino groups of RHAMM must be blocked. This is accomplished by dissolving the modified RHAMM peptide in potassium phosphate buffer at pH 7.5, followed by the addition of

25   citraconic anhydride dissolved in dioxane. The modified and blocked RHAMM peptide is then purified by SEC in 50 mM ammonium bicarbonate and lyophilized. The preparation of the RHAMM peptide for silver ion activation of the thiocarboxyl C-terminus is illustrated structurally below.

Boc-Gly-COSH   +   [Handle]-COOH

STEP 1:

$\downarrow$

Boc-Gly-COS-[Handle]-C-OH

STEP 2: $\qquad \downarrow$ DCC + H_2N-CH$_2$-Resin

5   Boc-Gly-COS-[Handle]-CONH-Resin

$\downarrow$

STEP 3: $\downarrow$ 1) RHAMM synthesis by Boc SPPS method

                  2) HF

H_2N^α-RHAMM-Gly-SH

$\downarrow$

STEP 4: $\downarrow$ Citraconic anhydride

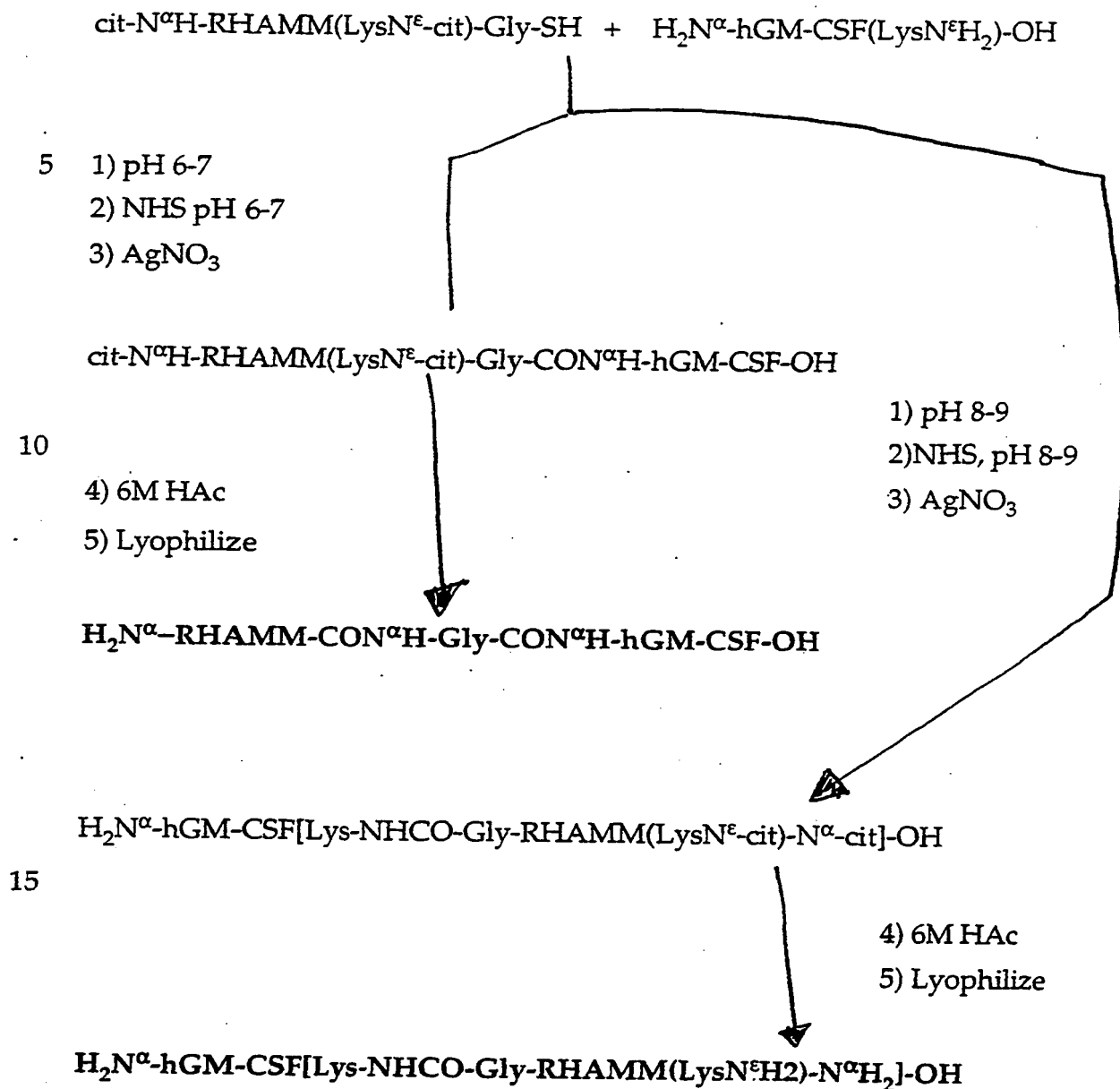10   cit-N^αH-RHAMM(LysN$^\epsilon$cit)-Gly-SH

Coupling of RHAMM peptide and hGM-CSF

For the coupling of the RHAMM peptide to the ε amino groups on the lysine residues of the protein, conjugation should take place at a pH of 8-9. If conjugation between the RHAMM peptide and predominantly the α

15   amino group (N-terminus) of the protein is preferred, conjugation should take place at pH 6-7.

Both peptide and protein are dissolved in water and pH adjusted as desired. This is followed by the addition of N-hydroxysuccinimide at 0°C (also at desired pH). AgNO$_3$ is added, and the solution is incubated at 0°C

20   for 30 minutes and at room temperature for 45 minutes (Blake and Li, 1981, 1983). The protective citraconyl groups are removed by incubation with a large excess of 6M acetic acid for 2.5 hours. The final product is lyophilized and purified by RP-HPLC using water/TFA and acetonitrile/TFA. The

product is eluted by buffer exchange into an appropriate buffer using SEC. The coupling reaction between hGM-CSF and prepared RHAMM peptide is illustrated structurally below.

cit-N$^\alpha$H-RHAMM(LysN$^\varepsilon$-cit)-Gly-SH + H$_2$N$^\alpha$-hGM-CSF(LysN$^\varepsilon$H$_2$)-OH

5   1) pH 6-7
    2) NHS pH 6-7
    3) AgNO$_3$

cit-N$^\alpha$H-RHAMM(LysN$^\varepsilon$-cit)-Gly-CON$^\alpha$H-hGM-CSF-OH

                                                1) pH 8-9
10   2) NHS, pH 8-9
    4) 6M HAc   3) AgNO$_3$
    5) Lyophilize

H$_2$N$^\alpha$–RHAMM-CON$^\alpha$H-Gly-CON$^\alpha$H-hGM-CSF-OH

H$_2$N$^\alpha$-hGM-CSF[Lys-NHCO-Gly-RHAMM(LysN$^\varepsilon$-cit)-N$^\alpha$-cit]-OH

15

                                                4) 6M HAc
                                                5) Lyophilize

H$_2$N$^\alpha$-hGM-CSF[Lys-NHCO-Gly-RHAMM(LysN$^\varepsilon$H2)-N$^\alpha$H$_2$]-OH

Characterization

20   The level of conjugation can be assessed by·determining the mass of the conjugate, and a tryptic digestion map will determine the point of substitution (if not N-terminal).

EXAMPLE 6

Conjugation of RHAMM peptide and parathyroid hormone (PTH) by the coupling of minimally protected peptides

Parathyroid hormone has been used therapeutically to increase bone mineral density in osteoporosis, corticosteroid-induced osteoporosis,
5 estrogen deficiency-related bone loss, and hypoparathyroidism. The conjugation of a PTH to RHAMM would exploit the distribution of endogenous HA, allowing RHAMM to direct PTH to its main target of therapeutic action, the bones. Administration of PTH has been associated with various nonspecific side effects, which may not be produced following
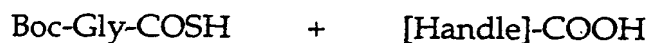10 administration of a PTH-RHAMM conjugate.

Preparation of recombinant PTH

The complete coding sequence of human PTH is given as GenBank Accession No. E01147, A08523, A08525 or A08533. For this method of coupling, the protein does not require modification or engineering prior to
15 the conjugation step. Recombinant PTH may be purchased from existing commercial sources, or produced by methods known to persons skilled in the art.
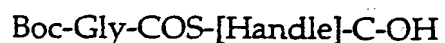
Preparation of RHAMM peptide

For the purpose of illustration in this example, the HA-binding
20 domain has an amino acid sequence as defined in SEQ. I.D. 13. It should be appreciated by those skilled in the art that other amino acid sequences representing other HA-binding peptides or polypeptides as contemplated hereunder shall constitute equivalent substitutions without departure from the principles of the present invention. The RHAMM peptide and is
25 synthesized with a C-terminal thiocarboxyl moiety, which may be accomplished by standard SPPS methods using the following procedure. Boc-thioglycine is coupled to a carboxyl handle, and then to an aminomethyl resin in the presence of dicyclohexylcarbodiimide (DCC) (Baca, 1995, Blake, 1981, and Yamashiro, 1988). The RHAMM peptide can then be synthesized
30 using standard Boc SPPS protocols and cleaved from the resin with HF to give RHAMM-Gly-thiocarboxyl. Prior to conjugation of the engineered RHAMM peptide with hGH, the amino groups of RHAMM must be blocked. This is accomplished by dissolving the modified RHAMM peptide

in potassium phosphate buffer at pH 7.5, followed by the addition of citraconic anhydride dissolved in dioxane. The modified and blocked RHAMM peptide is then purified by SEC in 50 mM ammonium bicarbonate and lyophilized. The preparation of the RHAMM peptide for silver ion
5   activation of the thiocarboxyl C-terminus is illustrated structurally below.

Boc-Gly-COSH   +   [Handle]-COOH

STEP 1:

Boc-Gly-COS-[Handle]-C-OH

↓
10   STEP 2:   DCC + H2N-CH2-Resin

Boc-Gly-COS-[Handle]-CONH-Resin

↓
STEP 3:   1) RHAMM synthesis by Boc SPPS method
                        2) HF

H$_2$Nα-RHAMM-Gly-SH

↓
15   STEP 4:   Citraconic anhydride

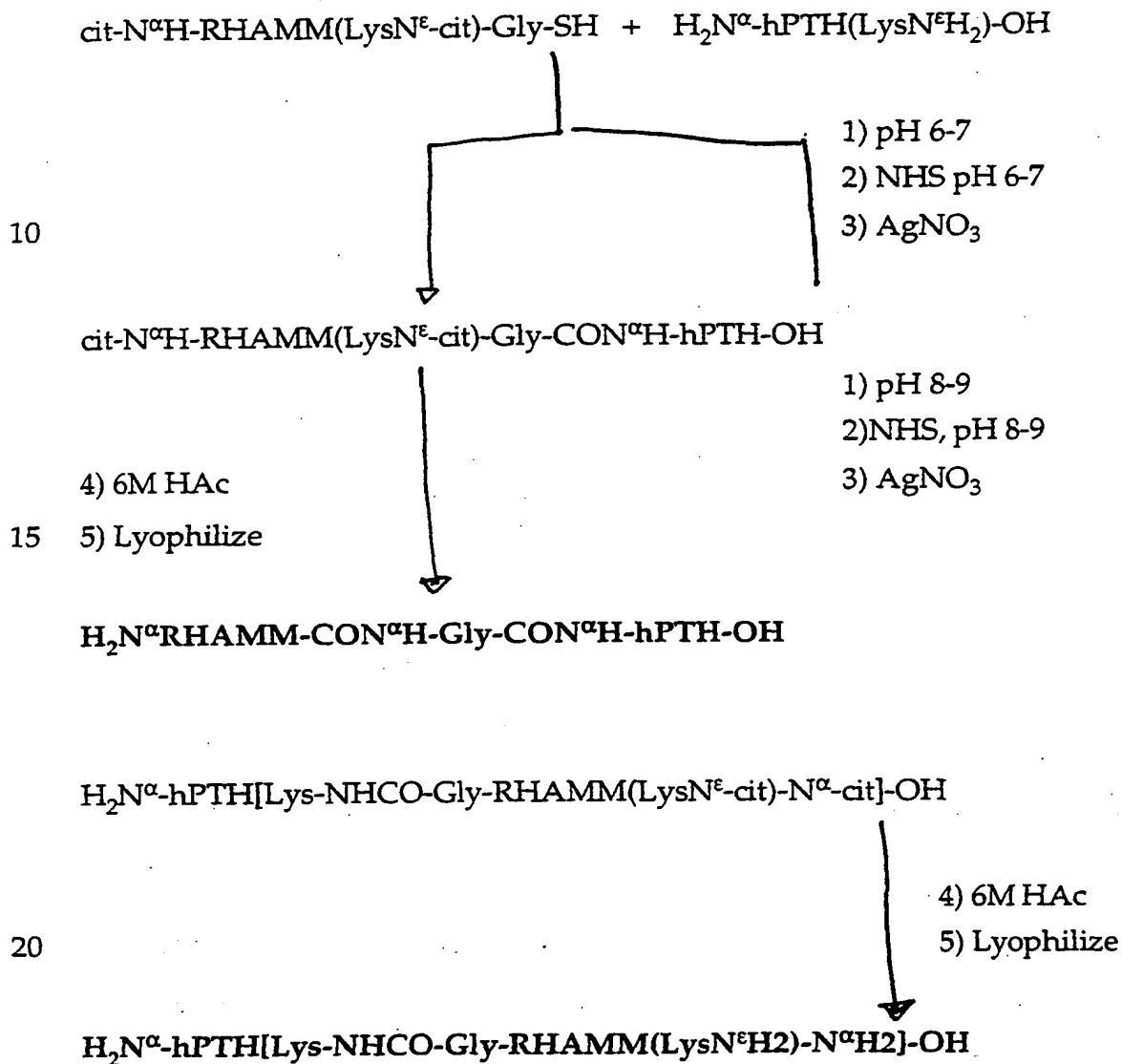cit-NαH-RHAMM(LysNϵcit)-Gly-SH

Coupling of RHAMM peptide and hPTH

For the coupling of the RHAMM peptide to the ϵ amino groups on the lysine residues of the protein, conjugation should take place at a pH of 8-
20   9. If conjugation between the RHAMM peptide and predominantly the α amino group (N-terminus) of the protein is preferred, conjugation should take place at pH 6-7.

Both peptide and protein are dissolved in water and pH adjusted as desired. This is followed by the addition of N-hydroxysuccinimide at 0°C
25   (also at desired pH). AgNO$_3$ is added, and the solution is incubated at 0°C for 30 minutes and at room temperature for 45 minutes (Blake and Li, 1981,

1983). The protective citraconyl groups are removed by incubation with a large excess of 6M acetic acid for 2.5 hours. The final product is lyophilized and purified by RP-HPLC using water/TFA and acetonitrile/TFA. The product is eluted by buffer exchange into an appropriate buffer using SEC.

5 The coupling reaction between hPTH and prepared RHAMM peptide is illustrated structurally below.

cit-N^αH-RHAMM(LysN$^\epsilon$-cit)-Gly-SH + H$_2$N$^\alpha$-hPTH(LysN$^\epsilon$H$_2$)-OH

1) pH 6-7
2) NHS pH 6-7

10   3) AgNO$_3$

cit-N^αH-RHAMM(LysN$^\epsilon$-cit)-Gly-CON$^\alpha$H-hPTH-OH

1) pH 8-9
2) NHS, pH 8-9

4) 6M HAc   3) AgNO$_3$

15   5) Lyophilize

H$_2$N$^\alpha$RHAMM-CON$^\alpha$H-Gly-CON$^\alpha$H-hPTH-OH

H$_2$N$^\alpha$-hPTH[Lys-NHCO-Gly-RHAMM(LysN$^\epsilon$-cit)-N$^\alpha$-cit]-OH

4) 6M HAc

20   5) Lyophilize

H$_2$N$^\alpha$-hPTH[Lys-NHCO-Gly-RHAMM(LysN$^\epsilon$H2)-N$^\alpha$H2]-OH

Characterization

The level of conjugation can be assessed by determining the mass of the conjugate, and a tryptic digestion map will determine the point of

25   substitution (if not N-terminal).

EXAMPLE 7

Production of RHAMM-Amantadine direct conjugate using thioether linkage

Preparation of amantadine

5   Amantadine was developed in the 1960s and has diverse therapeutic utiltities ranging from prevention of influenza A infection to the treatment of Parkinson's disease (Aoki and Sitar, 1988). 1-Aminoadamantane hydrochloride (amantadine hydrochloride) is available commercially as an antiviral under the name Symmetrel (E. I. du Pont de Nemours and

10   Company, Wilmington, Del.). Amantadine hydrochloride may also be prepared as known in the art, as described in U.S. Pat. No. 3,310,469.

       A sulfhydryl group must be added to the nitrogen of the primary amine group of amantadine for attachment of the RHAMM peptide. This may be accomplished by dissolving amantadine in 50 mM phosphate buffer

15   and 150 mM NaCl at a pH of 8. 2-iminothiolane (2-IT) is added at a 10-fold molar excess, followed by incubation for one hour at room temperature (Hermanson, 1996; Traut et al., 1973). Following thiolation, the thiolated amantadine is purified by SEC and lyophilized. The preparation of amantadine for thioether linkage conjugation to RHAMM is illustrated
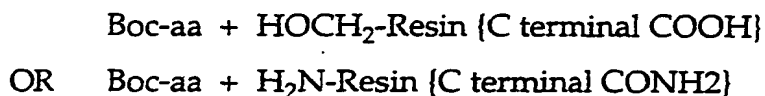
20   structurally below.

Amantadine-NH2

$\downarrow$

STEP 1:   1) pH8

                             2) 2-IT

25   Amantadine-NHC(=NH$_2$$^+$)CH$_2CH_2CH_2$-SH

Preparation of RHAMM peptide

       For the purpose of illustration in this example, the HA-binding domain has an amino acid sequence as defined in SEQ. I.D. 13. It should be appreciated by those skilled in the art that other amino acid sequences

30   representing other HA-binding peptides or polypeptides as contemplated

hereunder shall constitute equivalent substitutions without departure from the principles of the present invention. The RHAMM peptide must be engineered with an N-terminal bromoacetyl group, which will later be ligated to the thioloated primary amine. To this end, the RHAMM peptide is

5   coupled to a hydroxymethyl resin if a free acid is desired, or to a benzhydrylamine resin for a C-terminal amide. RHAMM is then synthesized as per normal Boc or Fmoc solid phase peptide synthesis (SPPS) methods. Following complete synthesis of the RHAMM peptide, the peptide is deprotected and coupled with bromoacetic anhydride (Robey,

10 1994; Robey and Fields, 1989). Finally, SEC and/or RP-HPLC methods are used to purify the peptide. The process is illustrated structurally below.
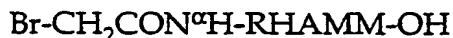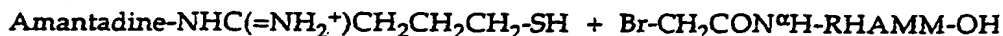
$$Boc\text{-}aa + HOCH_2\text{-}Resin \{C\ terminal\ COOH\}$$

$$OR \quad Boc\text{-}aa + H_2N\text{-}Resin \{C\ terminal\ CONH2\}$$

$$\downarrow$$

STEP 2:   1) Boc synthesis of RHAMM peptide

15   2) deprotect

                3) Bromoacetic acid anhydride

$$Br\text{-}CH_2CON^\alpha H\text{-}RHAMM\text{-}OH$$

*Coupling of the RHAMM peptide and amantadine*

Thiolated amantadine is dissolved in 0.1 M NaHCO$_3$. If solubility in

20 aqueous solution is problematic, this could be circumvented by firt dissolving the small molecule drug in a small percentage of organic acid. The solid bromoacetylated RHAMM peptide is added to the solution, the pH adjusted to 8, and the solution is allowed to incubate at room temperature for 3 hours (Robey and Fields, 1989, Kolodny and Robey, 1990, Muir et al.,

25 1994). Purification of the conjugate is by SEC and/or RP-HPLC. The structural representation of the conjugation reaction is illustrated below.

$$Amantadine\text{-}NHC(=NH_2^+)CH_2CH_2CH_2\text{-}SH + Br\text{-}CH_2CON^\alpha H\text{-}RHAMM\text{-}OH$$

$$\downarrow$$

STEP 3: $\downarrow$ 1) pH 8

2) incubate at room temperature 3h

3) purify by SEC and/or RP-HPLC

Amantadine-NHC(=NH$_2$)CH$_2$CH$_2$CH$_2$-S-CH$_2$CONH-RHAMM-OH
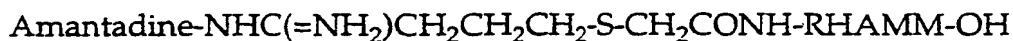
5 *Characterization*

The level of conjugation can be assessed by determining the mass of the conjugate, and a tryptic digestion map will determine the point of substitution.

EXAMPLE 8

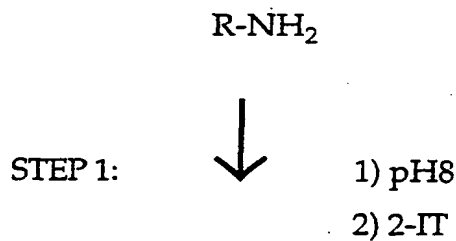10 Conjugation of HA-binding domain to antiviral agents using backbone engineered thioether linkage

Preparation of Acyclovir and Ganciclovir

Acyclovir (acycloguanosine, 2-Amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6H-purin-6-one) and ganciclovir (DHPG) are

15 synthetic acyclic purine nucleoside analogs with antiviral activity. Acycloviris sold under the tradename Zovirax™ (Glaxo Wellcome) for the treatment of herpes simplex, chicken pox and zoster infections while Ganciclovir is sold under the tradename Cytovene™ (Roche) for the treatment of cytomegalovirus infections. Both products are available

20 commercially and may also be sourced from Sigma (Sigma product numbers A250 and G2536 respectively). Acyclovir and ganciclovir may also be prepared as known in the art, as described in U.S. Pat. Nos. 4,544,634 and 5,994,321.
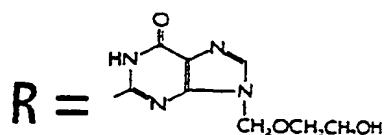
For the present example, a sulfhydryl group must be added to the

25 nitrogen of the amino group of acyclovir or gangiclovir for attachment of the RHAMM peptide. This may be accomplished by dissolving acyclovir or gangiclovir in 50 mM phosphate buffer and 150 mM NaCl at a pH of 8. If solubility in aqueous solution is problematic, this could be circumvented by firt dissolving the small molecule drug in a small percentage of organic acid.

30 2-iminothiolane (2-IT) is added at a 10-fold molar excess, followed by

incubation for one hour at room temperature (Hermanson, 1996; Traut et al., 1973). Following thiolation, the thiolated product is purified by SEC and lyophilized. The preparation of acyclovir or gangiclovir for thioether linkage conjugation to RHAMM is illustrated structurally below.
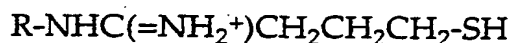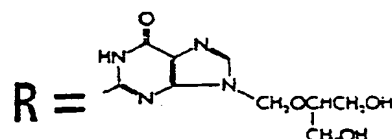
5

R-NH₂

STEP 1:   ↓   1) pH8
2) 2-IT

R-NHC(=NH₂⁺)CH₂CH₂CH₂-SH
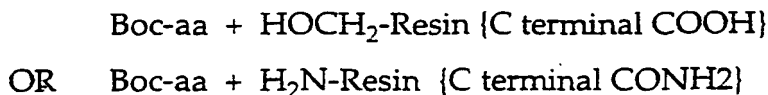
Acyclovir:

R =

Ganciclovir:

R =

10   *Preparation of RHAMM peptide*

For the purpose of illustration in this example, the HA-binding domain has an amino acid sequence as defined in SEQ. I.D. 13. It should be appreciated by those skilled in the art that other amino acid sequences representing other HA-binding peptides or polypeptides as contemplated

15   hereunder shall constitute equivalent substitutions without departure from the principles of the present invention. The RHAMM peptide must be engineered with an N-terminal bromoacetyl group, which will later be ligated to the thioloated lysine residues. To this end, the RHAMM peptide is coupled to a hydroxymethyl resin if a free acid is desired, or to a

20   benzhydrylamine resin for a C-terminal amide. RHAMM is then synthesized as per normal Boc or Fmoc solid phase peptide synthesis (SPPS) methods. Following complete synthesis of the RHAMM peptide, the peptide is deprotected and coupled with bromoacetic anhydride (Robey, 1994; Robey and Fields, 1989). Finally, SEC and/or RP-HPLC methods are

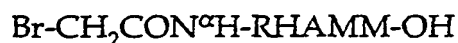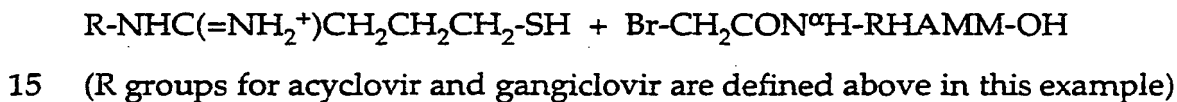25   used to purify the peptide. The process is illustrated structurally below.

Boc-aa + HOCH$_2$-Resin {C terminal COOH}

OR   Boc-aa + H$_2$N-Resin {C terminal CONH2}

STEP 2:   ↓   1) Boc synthesis of RHAMM peptide

2) deprotect

5   3) Bromoacetic acid anhydride

Br-CH$_2$CON$^{\alpha}$H-RHAMM-OH

Coupling of the RHAMM peptide and acyclovir or gangiclovir
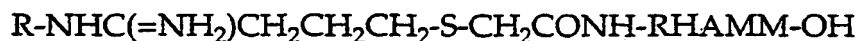
Thiolated acyclovir or gangiclovir is dissolved in 0.1 M NaHCO$_3$. The solid bromoacetylated RHAMM peptide is added to the solution, the pH

10   adjusted to 8, and the solution is allowed to incubate at room temperature for 3 hours (Robey and Fields, 1989, Kolodny and Robey, 1990, Muir et al., 1994). Purification of the conjugate is by SEC and/or RP-HPLC. The structural representation of the conjugation reaction is illustrated below.

R-NHC(=NH$_2$$^+$)CH$_2CH_2CH_2$-SH + Br-CH$_2CON^{\alpha}$H-RHAMM-OH

15   (R groups for acyclovir and gangiclovir are defined above in this example)

STEP 3:   ↓   1) pH 8

2) incubate at room temperature 3h

3) purify by SEC and/or RP-HPLC

R-NHC(=NH$_2$)CH$_2$CH$_2$CH$_2$-S-CH$_2$CONH-RHAMM-OH

20   *Characterization*

The level of conjugation can be assessed by determining the mass of the conjugate, and a tryptic digestion map will determine the point of substitution.

EXAMPLE 9

Conjugation of HA-binding domain to anti-cancer agent using direct thioether linkage

Preparation of methotrexate and cytarabine

5   Methotrexate, N -[4-[[(2,4-diamino-6-pteridinyl) methyl]methylamino]benzoyl]-L-glutamic acid, is one of the oldest chemotherapy drugs which is used alone or in combination with other chemotherapies, surgery or radiation for the treatment of cancers, lymphomas, leukemias, rheumatoid arthritis, and severe psoriasis.

10   Cytarabine, 4-amino-1-β-D-arabinofuranosyl-2 (1H)-pyrimidinone or Cytosine arabinoside, is indicated for remission induction in acute non-lymphocytic leukemia of adults and pediatric patients. It has also been found useful in the treatment of acute lymphocytic leukemia and the blast phase of chronic myelocytic leukemia. Intrathecal administration of CYTOSAR-U is

15   indicated in the prophylaxis and treatment of meningeal leukemia. Both products are available commercially and may also be sourced from Sigma (Sigma product numbers M9929 and C1768 respectively). Methotrexate and cytarabine may also be prepared as known in the art, as described in U.S. Pat. Nos. 4,080,325, 4,224,446, 4,374,987 and 5,641,758.

20   A sulfhydryl group must be added to the nitrogen of the amino group of methotrexate or cytarabine for attachment of the RHAMM peptide. This may be accomplished by dissolving methotrexate or cytarabine in 50 mM phosphate buffer and 150 mM NaCl at a pH of 8. If solubility in aqueous solution is problematic, this could be circumvented by

25   firt dissolving the small molecule drug in a small percentage of organic acid. 2-iminothiolane (2-IT) is added at a 10-fold molar excess, followed by incubation for one hour at room temperature (Hermanson, 1996; Traut et al., 1973). Following thiolation, the thiolated product is purified by SEC and lyophilized. The preparation of methotrexate or cytarabine for thioether

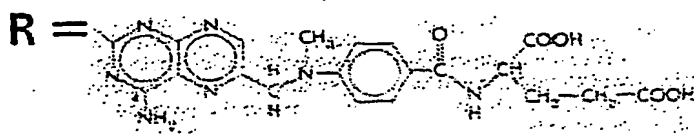30   linkage conjugation to RHAMM is illustrated structurally below.

$$R\text{-}NH_2$$

STEP 1: 1) pH8
         2) 2-IT

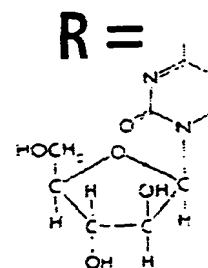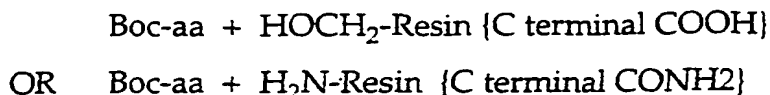$$R\text{-}NHC(=NH_2^+)CH_2CH_2CH_2\text{-}SH]\text{-}OH$$
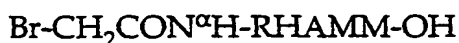
Methotrexate:

Cytarabine:

5

Preparation of RHAMM peptide

For the purpose of illustration in this example, the HA-binding domain has an amino acid sequence as defined in SEQ. I.D. 13. It should be appreciated by those skilled in the art that other amino acid sequences

10 representing other HA-binding peptides or polypeptides as contemplated hereunder shall constitute equivalent substitutions without departure from the principles of the present invention. Furthermore, the following method is a representation of a multitude of methods as known in the art for conjugation of a polypeptide to an anti-cancer agent, for example, as

15 described in U.S. Pat. No. 5,108,987.

In this instance, the RHAMM peptide must be engineered with an N-terminal bromoacetyl group, which will later be ligated to the thioloated lysine residues. To this end, the RHAMM peptide is coupled to a hydroxymethyl resin if a free acid is desired, or to a benzhydrylamine resin

20 for a C-terminal amide. RHAMM is then synthesized as per normal Boc or Fmoc solid phase peptide synthesis (SPPS) methods. Following complete synthesis of the RHAMM peptide, the peptide is deprotected and coupled with bromoacetic anhydride (Robey, 1994; Robey and Fields, 1989). Finally, SEC and/or RP-HPLC methods are used to purify the peptide. The process

25 is illustrated structurally below.

Boc-aa + HOCH$_2$-Resin {C terminal COOH}

OR   Boc-aa + H$_2$N-Resin {C terminal CONH2}

$\downarrow$

STEP 2:   1) Boc synthesis of RHAMM peptide

2) deprotect

5   3) Bromoacetic acid anhydride

Br-CH$_2$CON$^\alpha$H-RHAMM-OH

Coupling of the RHAMM peptide and methotrexate or cytarabine

Thiolated methotrexate or cytarabine is dissolved in 0.1 M NaHCO3.
The solid bromoacetylated RHAMM peptide is added to the solution, the pH
10   adjusted to 8, and the solution is allowed to incubate at room temperature
for 3 hours (Robey and Fields, 1989, Kolodny and Robey, 1990, Muir et al.,
1994). Purification of the conjugate is by SEC and/or RP-HPLC. The
structural representation of the conjugation reaction is illustrated below.

R-NHC(=NH$_2$$^+$)CH$_2CH_2CH_2$-SH + Br-CH$_2CON^\alpha$H-RHAMM-OH
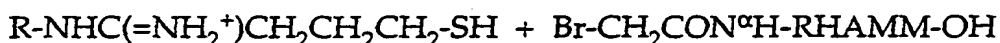
15   (R groups for methotrexate and cytarabine are defined above in this
example)

$\downarrow$

STEP 3:   1) pH 8

2) incubate at room temperature 3h

3) purify by SEC and/or RP-HPLC

20   R-NHC(=NH$_2$)CH$_2$CH$_2$CH$_2$-S-CH$_2$CONH-RHAMM-OH
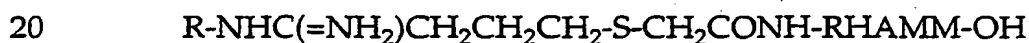
Characterization

The level of conjugation can be assessed by determining the mass of
the conjugate, and a tryptic digestion map will determine the point of
substitution.

EXAMPLE 10

The *in vitro* HA-binding potency of any RHAMM conjugate can be assessed using an activated sepharose column. The sepharose is prepared according to the manufacturer's instructions and mixed with HA, resulting in a medium that can be used as an affinity column. The radiolabelled RHAMM conjugate is passed through the column, and the column is washed. If the HA-binding site of the RHAMM peptide conjugate remains accessible to HA, the conjugate will remain bound to the sepharose and will not be present in the eluent. The fraction of RHAMM peptide that remains bound to the column can be calculated based on the radioactivity of the column and eluent following washing of the column with buffer. This result is then compared with the results of passing unconjugated radiolabelled RHAMM peptide through an identical column, to determine the HA-binding ability of the conjugate.

EXAMPLE 11

In addition to the previous experiment, the potency of the RHAMM conjugate can be further determined by a competitive binding experiment. The column is prepared by mixing the activated sepharose with hyluronan, and RHAMM peptide is then added to the column along with the radiolabelled RHAMM conjugate. In this manner, the potency of the conjugate can be compared with that of RHAMM peptide alone.

The pharmacokinetics and distribution of the RHAMM-protein conjugates can be determined in rabbits, rats, mice, or other animals by various methods known to persons skilled in the art. One experimental design may involve the parenteral injection of the conjugate molecule into rabbits and mice at predetermined doses. Twelve rabbits are to be included in the preliminary intravenous study to evaluate *in vivo* profiles of the conjugate molecules, with 6 rabbits in each group. The concentrations of radiolabelled test peptides in plasma will be determined at 5, 15, 30, and 45 minutes, and at 1, 2, 3, 6, 12, and 24 hours following intravenous administration by radioactivity counting.

The single dose study will be carried out in 2 groups of mice, one group allocated to intravenous injection, and one group to intramuscular

injection. Sampling times will be determined based on results from the preliminary study in rabbits. Five test animals will be sacrificed at each sampling time and blood and organs will be collected. Urine and feces will also be collected in metabolic cages. The concentrations of the conjugate in various tissues will be analyzed by radioactivity counting.

A peptide having an identical amino acid composition to the conjugate will be administered under the same protocol to a third group of mice. The results from this experimental group will be compared to that of the animals receiving the conjugate molecule.

All animals will be euthanized after the study, and various organs (liver, kidney, heart, spleen, brain, lung, and bones) collected for drug distribution analysis. Basic pharmacokinetic parameters including elimination half-life, clearance, and volume of distribution will be calculated following intravenous injection using standard equations and methods known to those persons in the field of pharmacokinetics. The parameters determined following intramuscular injection shall include elimination half-life, clearance, volume of distribution, peak concentration and time, and bioavailability.

EXAMPLE 12

The biological activity of GMCSF or a GMCSF-RHAMM conjugate can be determined by an *in vitro* bioassay using the TF-1 cell line and the MTT cell proliferation method. TF-1 cells areused in this assay, as it is a cell line of immature erythroid origin that proliferates dependently on GM-CSF.

One day prior to bioassay, the number of TF-1 cells necessary for growth factor deprivation must be calculated (4×10^4 cells are needed/assay well). Cells are centrifuged at 270xg for 5 minutes and resuspended in culture media. This process is repeated, the supernatant aspirated, and the cells are resuspended in culture media to a concentration of 2×10^5 cells/mL. The cell suspension is incubated at 37°C, 8%CO_2 for 20-24 hours. Culture media can be prepared by dissolving 50ml heat-inactivated fetal bovine serum (HI-FBS) in 500ml DMEM (Gibco catalogue# 11965-050), with 10μl 2.5M 2-mercaptoethanol.

On day zero of the bioassay, the viable cell count of the growth factor-deprived cells is determined, and the volume of cells necessary for the

assay is obtained (4×10^4 cells/assay well). The cells are centrifuged at 270xg for 5 minutes, followed by aspiration of the supernatant and resuspension of the cell pellet in 2 volumes of fresh assay medium. Cells are again centrifuged, the supernatant is aspirated, and the pellet is resuspended in

5 assay medium to a concentration of 4×10^5 cell/mL.

For preparation of the standard curve, the GMCSF standard is diluted with assay medium to a concentration of 600 IU/mL, and serial dilutions are performed for determination of the standard curve. Assay medium is prepared in RPMI without phenol red (Gibco catalogue# 11835-030), to a

10 final concentration of 1mg/ml insulin, 1 mg/ml transferrin, and 1×10^{-4}M 2-mercaptoethanol. Insulin and transferrin stock solutions are diluted first in Dulbecco's phosphate buffered saline (D-PBS) to 25 mg/ml, and 2-mercaptoethanol is mixed with gentamicin to 0.05M. Each test sample is prepared and diluted appropriately with sterilized PW to obtain a protein

15 concentration of 0.4 - 0.6 mg/ml. The protein concentration is then calculated, and the process is repeated as required to obtain a dilution which gives a protein concentration of 0.4 - 0.6 mg/mL. This sample is then diluted 50x with 0.1% BSA in D-PBS. This will result in a GM-CSF concentration of approximately 10ng/mL.

20 Eight 2-fold serial dilutions of the GM-CSF samples in assay medium are performed for determination of the dose-response curve. For the assay, approximately 4×10^4 cells are added to each well with 50ml of sample, standard, or blank. The plates are incubated at 37°C, 5%CO_2 for 72 hours. After incubation, MTT solution (4.0 mg/ml in D-PBS) is added to a

25 concentration of 2 mg/ml, and the plate is sealed and mixed for 30 seconds, followed by incubation for 3 hours at 37°C, 5%CO_2. After incubation, the plate is centrifuged at 1775xg for 15 minutes. The supernatant is aspirated from all plates and 50ml isopropanol is added to each well, followed by mixing of the sealed plates for 5 minutes. Optical density is read at 595 nm.

30 The final potency of the sample is then calculated as follows:

Potency of test sample = $\dfrac{\text{IU giving 50\% maximal response (7.9)}}{\text{mg of test sample giving 50\% max. response (7.15)}}$

(IU/mg)

EXAMPLE 13

The biological activity of EPO samples can be determined by an *in-vitro* bioassay using the EPO dependant UT-7 cell line and the MTT cell proliferation method (Komatsu et al., Blood, vol 82, No. 2 (July 15), 1993: pp 456-464). UT-7 cells (human leukemic cell line) are grown in UT-7 Culture Media supplemented with feed EPO to give a final EPO concentration of 1U/mL. This medium can be prepared by mixing 500 mL of RPMI-1640 media (Gibco catalogue #11875-093 or equivalent), 50 mL of HI-FBS (Gibco catalogue #10438-026 or equivalent), 10mL of 2.5M 2-mercaptoethanol (174mL 2-mercaptoethanol, ACS grade or equivalent + 826mL of D-PBS, Gibco cat# 14040-026 or equivalent. Sterile filtered using a 0.2mm syringe filter) in a laminar flow hood. Then sterile filter into a sterile 500mL bottle using a 0.2mm bottle-top filter.

Cells should be subcultured or fed every 48-96hrs. Subculturing concentrations can range from 0.5×10^5 - 2×10^5 cells/mL depending on the length of time before the next subculture or feeding. The cells are to be incubated at 37°C in a 5% CO_2 atmosphere. On the first day of experimentation, UT-7 cells are deprived of growth factor. Cells are counted to determine cell concentration, with a minimum of 4×10^4 cells needed per assay well. Cells are removed as needed and placed in a sterile centrifuge tube for centrifugation at 270g for 5 minutes. The supernatant is then aspirated and the cells are resuspended in half the volume of UT-7 culture media, followed by centrifugation of cells again at 270g for 5 minutes. The resulting supernatant is aspirated cells are resuspended to a cell seeding concentration of 2×105 cells/mL. Following transfer of the cell suspension to T-75 culture flasks, cells are incubated at 37°C, with 5% CO_2 for 20-26hrs.

The second experimental day involves preparation of cells for use in the assay and plating of standard and samples. The cells are centrifuged at 270g for 5 minutes, the supernatant is aspirated, and cells are resuspended in half the volume of assay media. Assay medium is prepared by mixing together 200ml of 25mg/mL insulin [insulin (Boehringer Mannheim catalogue # 1376497) dissolved in sterile, filtered D-PBS, pH 3.0], 500mL of RPMI-1640 without phenol red (Gibco cat# 11835-030 or equivalent), 200mL of 25mg/mL transferrin (Boehringer Mannheim cat#1073974 or equivalent) in D-PBS, 20mL of 2.5M 2-mercaptoethanol, 1mL of 10mg/mL gentamicin

(Gibco cat#15710-023 or equivalent). Assay medium can be stored at 2-8°C. The suspension should result in an approximate cell concentration of 4x105cells/ml. Cells can now be returned to a 37°C, 5% CO2 incubator until needed (up to 3 hrs).

5   Dilute the EPO standard (R&D Systems Inc. Catalogue #287-TC reconstituted in 0.1% BSA in D-PBS) in the assay medium to a concentration of 20U/mL, and add 100mL of the 20U/mL EPO standard to wells A1, B1, and C1 of a 96 well plate. Add 50mL of assay media to wells A2, B2, C2 through A12, B12 and C12 inclusive. Serial dilution is performed by
10   removing 50mL from wells A1, B1, and C1 and transferring it to wells A2, B2, and C2. This series dilution is continued until wells A10, B10, and C10 are reached at which point 50mL are removed from these wells and discarded. Wells A11 through C12, inclusive, are blank wells and should contain only 50mL of assay media at this time.

15   Samples are prepared by dilution in 0.1%BSA in D-PBS to a concentration at which they can be diluted at least a further 1/10 in assay media prior to addition to the assay plate. $100\mu L$ of sample dilution is added to the sample/unknown plate in triplicate, followed by addition of $50\mu L$ assay media to wells in which the sample will be serially diluted. Serial
20   dilution of the sample is performed by removing $50\mu L$ from the starting wells and transferring this to the next set of wells. This is repeated until appropriate dilution range is obtained and then $50\mu L$ of sample/unknown dilution from the last set of wells is discarded.

        UT-7 cells are removed from the incubator and resuspended,
25   followed by transfer of cells to a sterile petri dish. 100mL of cells is added to all standard and sample/unknown wells, and plates are incubated at 37°C, 5% CO_2 for 44-76hrs.

        Following the incubation, $50\mu L$ of 4.0mg/mL MTT [3 - (4, 5 - Dimethylthiazolyl - 2) - 2, 5 - Diphenyltetrazolium Bromide] (Sigma
30   catalogue #M5655 in 50 ml D-PBS) is added to each standard and sample/unknown well. Plates are agitated on a plate shaker for approximately 30 seconds and then returned to the incubator for 2.5-4hrs. Plates are centrifuged at 3250rpm for 15 minutes, and supernatant aspirated. 50mL of isopropanol is added to all standard and sample/unknown wells,
35   followed by agitation of sealed plates for 5 minutes. Absorbance is read at

595nm.

The log dose-response curves for the standard and samples are plotted, the 50% of maximum O.D. is calculated, and linear regression is performed. Using the shared slope between the sample and the standard and the y-intercept for the standard curve, the EPO concentration of the standard which corresponds to the 50% O.D. is calculated and the quantity of EPO which results in the 50% O.D. Potency of the sample unknown is then calculated as follows:

$$\frac{\text{EPO (U) at 50\% maximum response O.D from the standard}}{\text{EPO (}\mu\text{g) of sample at 50\% maximum response O.D}}$$

EXAMPLE 14

The hGH activity of the hGH RHAMM conjugate can be determined by bioassay using the Nb2 cell line. The Nb2 rat lymphoma cell line was originally derived from a transplantable lymphoma which arose in the lymph nodes of an estrogenized male rat of the Noble (Nb) strain. The growth of Nb2 cells is dependent upon the presence of lactogenic hormones such as prolactin or growth hormone (Gout, P., Beer, C. & Noble, R. 1980. *Cancer Research* 40: 2433-2436; Tanaka, T., Shiu, R., Gout, P., Beer, C., Noble, R. & Friesen, H. 1980. *J. Clin. Endocrin. Metab.* 51: 1058-1063).

Standard rhGH is dissolved in purified water to a concentration of 6.0 IU/mL (2 mg/mL). This is further diluted in hGH dilution buffer to a concentration of 400 nIU/mL (133.3 ng/mL). For each two-plate bioassay, dilute this hGH solution further in Fisher's hGH assay medium to a concentration of 8000 nIU/ml.

hGH dilution buffer is prepared by dissolving 2.0 g glycine, 0.2 g mannitol, 0.2 g lactose, and 0.25 sodium bicarbonate in 0.80ml purified water. Then bovine serum albumin is dissolved to 0.5%, and purified water added to final volume. Fisher's hGH assay medium can be prepared by combining heat-inactivated horse serum (to 10%) and 2-mercaptoethanol (to 0.1mM) in Fisher's medium. Fisher's medium is a solution of 10% heat-inactivated horse serum (HI-HIS), 10% heat-inactivated fetal bovine (HI-FBS) serum, in Fisher's medium (FM).

The Nb2-11 cells are seeded in Fisher's medium and placed in a 37.0 ± 2.0°C / 5.0 ± 0.5% CO2 humidified incubator. Cells must be subcultured

before the concentration reaches 1×10^6 cells/ml to prevent the adaptation of cells to growth in HI-HS alone or development of a heterogeneous hGH growth-dependent cell population (approximately every 72 - 96 hours).

Nb2-11 cells are suspended in FM at a concentration of 1×10^5 cells/mL. A growth factor deprivation flask and control are prepared using a volume of cell suspension containing 1×10^6 cells for preparation of 10 mL at a concentration of 1×10^5 cells/mL. The flask is then incubated at $37.0 \pm 2.0°C$ / $5.0 \pm 0.5\%$ CO_2 until the cell count in the control flask plateaus (approximately 96-120 hours).

The cells are centrifuged at 270xg for 5 minutes, washed in assay medium, and recentrifuged. The supernatant is discarded ad the cells are resuspended in assay medium at a concentration of 8×10^6 cells (8×10^5 cells/mL). Following incubation at $37.0 \pm 2.0°C$ / $5.0 \pm 0.5\%$ CO_2 for 24.0 ± 2.0 hours, the cells will be in stationary phase.

During the bioassay, blank wells contain $100\mu L$ of FhAM + $100\ \mu L$ of cells + tracer and are used to monitor the uptake of tracer (i.e. cell proliferation) in the absence of rhGH and to provide the minimum response value for calculation of the 50% maximal response.

Serial dilutions are made of rhGH standard and samples following determination of sample protein concentration. The contents of the stationary flasks are pooled, and cells counted. Cell concentration is adjusted to 4×10^5 cells/mL by the addition of assay medium. A volume of 20mL is normally prepared for each bioassay plate to ensure an excess of cell suspension. This volume can be reduced to a minimum of 12.0mL per plate if there is a limiting number of cells. The prepared cells are transferred into a tissue culture dish at a maximum volume of 40 mL per dish and mixed. 100mL of the cells are added at a concentration of 4×10^5 cells/mL (4×10^4 cells/well) to all wells taking care to mix the cells prior to each uptake by gently stirring. The plates are incubated at $37.0 \pm 2.0°C$ / $5.0 \pm 0.5\%$ CO_2 for 70 hours.

Cells are labelled with 20 mL of tracer (1/100 ^3H-methyl-thymidine in assay medium) in each well, followed by incubation for 2.0 ± 0.25 hours at $37.0 \pm 2.0°C$ / $5.0 \pm 0.5\%$ CO_2.

Cells are harvested from the plates onto filter discs, washed, and placed into scintillation vials with 5 mL of scintillation fluid for counting. A

regression analysis is performed on the standard and samples, which will be compared to determine parallelism. The potency of the sample can be determined by the following equation:

$$\frac{EC^{std}_{50} \; (nIU/ml)}{\text{concentration of sample aliquot with same response as } EC^{std}_{50} \; (pg/ml)}$$

5

EXAMPLE 15

To investigate the effect of the RHAMM-hGH conjugate on body weight gain, hypophysectomized male rats will undergo daily subcutaneous injection for a minimum of 10 consecutive days. Male Sprague-Dawley
10 hypophysectomized rats (6 weeksof age, 70-90 g) will be purchased and shipped to the animal care facility. Following arrival, each animal will be given a general physical examination by a member of the veterinary staff to assess health status. An acclimation period of approximately 2 weeks will be allowed between animal receipt and the start of treatment in order to
15 accustom the animals to the laboratory environment. Animals will be housed individually at 22 ± 3°C with a 12 hour light/dark cycle in stainless steel wire mesh-bottomed cages equipped with an automatic watering valve. Municipal tap water which has been softened, purified by reverse osmosis and sterilized by ultraviolet light will be freely available (except
20 during designated procedures). All animals will have free access to a standard certified pelleted commercial laboratory diet (PMI Certified Rodent Chow 5002: PMI Feeds Inc.) except during designated procedures.

Approximately 1 week before treatment initiation, all animals will be weighed and assigned to treatment groups using a computer generated
25 randomization procedure. There will be 7 treatment groups each with 16 animals, consisting of one control group (0.9% saline solution), three groups receiving hGH at doses of 10, 50, and 100μg/rat/day, and three groups receiving the RHAMM-hGH conjugate at doses to be determined based on preliminary studies. The intrascapular injections will be made in a volume
30 of 1 mL, and shall be administered once daily for 9 consecutive days. During the first 5 days of treatment, animals will have free access to a water bottle containing 5% Dextrose, U.S.P. During the last 4 days of treatment, the solution will be reduced to a concentration of 2.5% Dextrose, U.S.P.

All animals will be examined twice daily for mortality and signs of ill-

health or reaction to treatment. Death and observed clinical signs will be individually recorded. Individual body weights will be measured daily for at least 10 days prior to treatment and continuing throughout the treatment period. Following 10 days of treatment (i.e. on day 11), the animals will be
5 euthanized by carbon dioxide asphyxiation and the carcasses discarded without further examination.

The osteogenic effects of RHAMM-protein conjugates (eg. RHAMM-hGH, RHAMM-PTH) must be determined *in vivo*. The RHAMM-protein conjugate or inactive protein conjugate will be administered to 2 groups of
10 rats, respectively, at dose schedules and intervals to be determined by preliminary studies (5 days/week for 2 months). Peripheral quantitative computed tomography (pQCT) will be used to measure bone mineral content, bone density, and geometric parameters of the right distal femur *ex vivo*. Femur was selected since it is easily scanned and is relatively straight,
15 and increases in bone mineral content and bone area following GH treatment have been documented in femur by this method (Banu, J.,Orhii, P.B., Okafor, M.C., et al., 1999). In addition, parathyroid hormone has also been shown to increase femoral bone mineral density and bone area, and the combination of GH and PTH increases bone strength more than either
20 therapy alone (Wang, L., Orhii, P.B., Banu, J., and Kalu, D.N., 1999).

pQCT scans will be performed using a Norland XCT Research SA bone scanner with software version 5.40. A single scan will be obtained of the femoral metaphysis at approximately 18% of the bone length as measured from the distal end of the femur. In addition, a second scan will
25 be acquired at 50% of the bone length. The pQCT procedure will characterise any changes in bone geometry and bone density due to treatment. Distal femoral scans will be evaluated for area, bone mineral content, and bone density of the total slice and the trabecular and cortical/subcortical regions, periosteal circumference and endosteal
30 circumference.

In addition to these endpoint parameters, biochemical markers of bone turnover will be analyzed pre- and post-treatment, such as osteocalcin, bone-specific alkaline phosphatase, and PTH in blood, and deoxypyridinoline, N-telopeptide, and creatinine in urine.
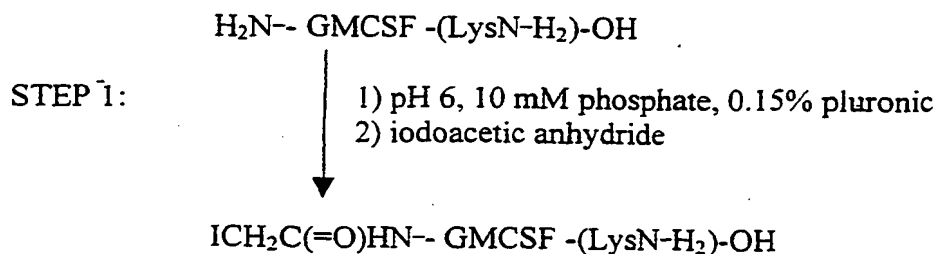
EXAMPLE 16

Production of RHAMM-GMCSF direct conjugate using backbone engineered thioether linkage

GMCSF would benefit substantially from the distribution of HA in bone
marrow, as targeted by RHAMM peptides. Recombinant erythropoietin is
currently produced in the glycosylated form, as the naked form is readily degraded
and, therefore, therapeutically ineffective. Therefore, conjugation of non-
glycosylated GM-CSF to a RHAMM peptide will increase the half-life of GM-CSF
and direct the conjugate to tissues with a high concentration of HA. It has been
reported that approximately 25% of HA is found in bone, which is an optimal target
for GM-CSF, and as such, in addition to increasing half-life, conjugation to
RHAMM may enhance target specificity.

Preparation of recombinant GMCSF

The complete coding DNA sequence of human GM-CSF is given as
GenBank Accession No. E00951. Recombinant GM-CSF may be purchased from
existing commercial sources, or produced by methods known to persons skilled in
the art.

A iodoacetyl group must be added to the N-terminal of GMCSF for
attachment of the RHAMM peptide. This may be accomplished by adjusting a
GMCSF solution (originally in 10 mM phosphate, pH 6.5) to 60mM phosphate
buffer and 0.15% pluronic at a pH of 6.0. Iodoacetic anhydride is added to a final
10-fold molar excess, i.e., an equimolar quantity is added in 10 aliqouts at 5 minute
intervals at room temperature. Following iodoacetylation, the protein is purified by
gel filtration on a PP10 coulumn. The preparation of recombinant GMCSF for
thioether linkage conjugation to RHAMM is illustrated structurally below, where
H2N- and —OH represent the N and C terminals of GMCSF, respectively.

$$H_2N- GMCSF -(LysN-H_2)-OH$$

STEP 1:

1) pH 6, 10 mM phosphate, 0.15% pluronic
2) iodoacetic anhydride

$$ICH_2C(=O)HN- GMCSF -(LysN-H_2)-OH$$

Preparation of RHAMM peptide

SEQ. I.D. 3 comprises the preferred sequence of RHAMM peptide for conjugation to EPO. The RHAMM peptide must be engineered with an C-terminal Cysteine group, which will later be ligated to the iodoacetylated N-terminal. To this end, a benzhydrylamine resin is utilized to produce a C-terminal amide and the first residue coupled to the resin is the amino acid Cysteine. RHAMM is then synthesized as per normal Boc or Fmoc solid phase peptide synthesis (SPPS) methods. Finally, SEC and/or RP-HPLC methods are used to purify the peptide. The process is illustrated structurally below.

$$Fmoc-Cys + H_2N-Resin \quad \{C\ terminal\ CONH_2\}$$

STEP 2:

1) Fmoc synthesis of RHAMM peptide
2) deprotect

$$N-H_2-RHAMM-Cys- CONH_2$$

Coupling of the RHAMM peptide and GMCSF

Iodoacetylated GMCSF is buffer exchanged into 10 mM phosphate, pH 7.0. The RHAMM-Cys peptide is reconstituted in 10 mM phosphate, pH 7.0 and added to the Iodoacetylated GMCSF solution. The solution is allowed to incubate at room temperature for 30 minutes (reference if any). Purification of the conjugate is by performed by RP-HPLC followed by SEC. The structural representation of the conjugation reaction is illustrated below.

ICH$_2$C(=O)HN-- GMCSF -(LysN-H$_2$)-OH + N-H$_2$-RHAMM-Cys- CONH$_2$

STEP 3:

1) pH 7.0
2) incubate at room temperature 30 minutes
3) purify by RP-HPLC followed by SEC

N-H$_2$-RHAMM-CH-(CONH$_2$)-CH$_2$-S-CH$_2$C(=O)HN-- GMCSF-OH

5
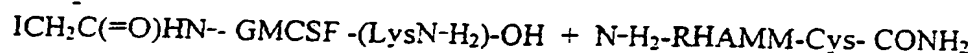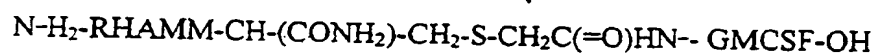
Characterization

The level of conjugation can be assessed by determining the mass and amino acid composition of the conjugate and a tryptic digestion map can verify the conjugation site.

10   *Diagramatic representation of reaction steps*

1. Modify protein with sulfo-GMBS:

2. Synthesize peptide with SAMA N-terminal:

3. Deprotect peptide with hydroxylamine:
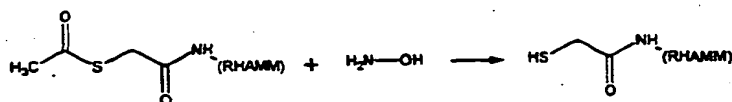
4. Link modified protein to deprotected peptide:
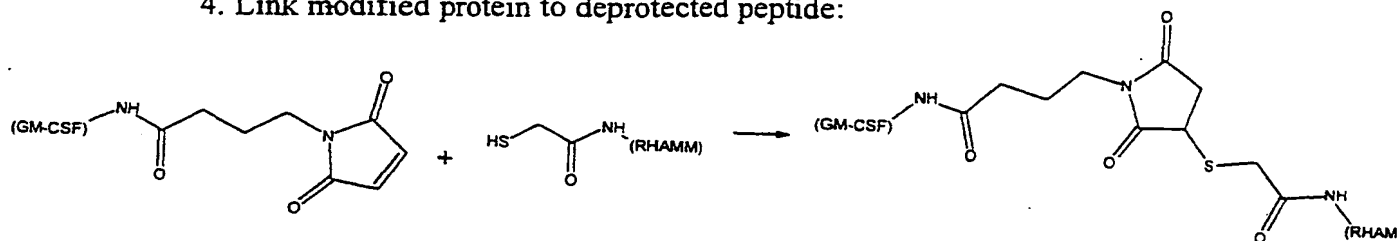
Characterization of RHAMM-GM-CSF Conjugate

The following describes the amino acid analysis of peptides and proteins using precolumn derivatization with PITC to form the PTC amino acids.

Materials: Chemicals

Constant boiling 6N hydrochloric acid (sequenal grade) *(Pierce/Chromatographic Specialties Cat.# 24309)*
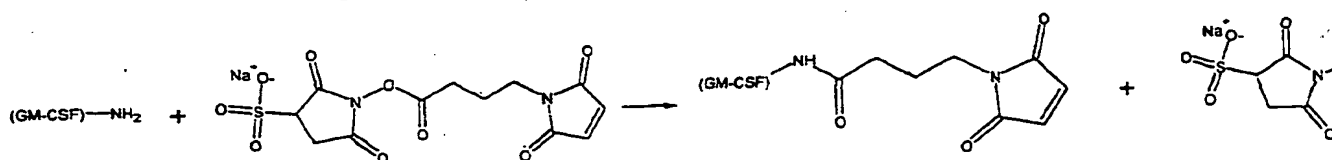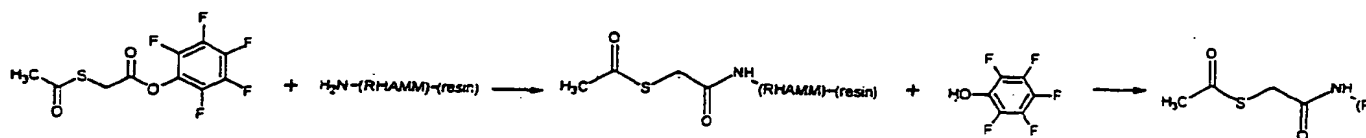
Amino acid standard H, 2.5 μmole/mL (Pierce/Chromatographic Specialties Cat.# 20088)

Type I reagent grade purified water or equivalent

Methanol (HPLC grade) *(Caledon Cat.# 6701-7 or equivalent)*

Phenylisothiocyanate, 1.13 g/mL (FW = 135.2) (sequenal grade) *(Pierce/Chromatographic Specialties Cat.# 26922)*

Triethylamine, 10 x 2 mL ampoules (sequenal grade) *(Fluka Cat. #90338; distributed through Sigma Chemical Co.)*

Glacial acetic acid (ACS reagent) *(Fisher Scientific Co. Cat.# A38-4 or equivalent)*

Sodium acetate, anhydrous (NaC$_2$H$_3$O$_2$, FW = 82.03) (ACS reagent) *(Sigma Chemical Co. Cat.# S-5889 or equivalent)*

Sodium phosphate dibasic heptahydrate (Na$_2$HPO$_4$ · 7H$_2$O, FW = 268.07) (ACS grade) *(BDH Cat.# ACS810 or equivalent)*

Acetonitrile-190 (CH$_3$CN) (HPLC grade) *(Caledon Cat.# 1401-7 or equivalent)*

Prepurified nitrogen (T-size and lecture bottle size) *(Medigas Cat.# 105 395)*

Phenol (C$_6$H$_6$O) (SigmaUltra) *(Sigma Chemical Co. Cat.# P-5566 or equivalent)*

S-Carboxymethyl-$_L$-cysteine (FW = 179.2) *(Sigma Chemical Co. Cat.# C-7757)*

O-phosphoric acid, 85% (HPLC grade) *(Fisher Scientific Co. Cat.# A260-500 or equivalent)*

Standard peptide, Ac-RGVGGLGLGK-amide; 0.069 mg/vial or equivalent concentration by AAA (API)

Materials: Equipment

Pico-Tag work station *(Waters Chromatography Division Cat.# 07370)*

5   Refrigerated condensation trap (RT100) *(Fisher Scientific Co.)*

Microfuge *(Eppendorf or equivalent)*

Ultrasonic bath #FS9 *(Fisher Scientific Company Cat.# 15-336-2 or equivalent)*

Thermolyne #1400 furnace *(Fisher Scientific Company Cat.# 10-552)*

Vacuum pump, Leybold D2A *(Fisher Scientific Company Cat.# 01-058-6 or*

10 *equivalent)*

Gilson pipetman: P20 (20 µL) *(Mandel Cat.# GF-23600 or equivalent)*, P200 (200 µL) *(Mandel Cat.# GF-23601 or equivalent)*, P1000 (1000 µL) *(Mandel Cat.# GF-23602 or equivalent)*

Disposable glass culture tubes (16x100 mm)*(Fisher Scientific Company Cat.#*

15 *14-961-29)*

Disposable glass culture tubes (12x75 mm) *(Fisher Scientific Company Cat.# 14-961-26)*

Pyrex culture tubes with screw cap (16x100 mm) *(Fisher Scientific Company Cat.# 14-930-13B)*

20 Screw cap for 4.2.10 with teflon inserts *(Fisher Scientific Company Cat.# 14-930-15E)*

Graduated glass cylinder (500 mL) *(Fisher Scientific Company Cat.# 08-549-5H or equivalent)*

Microfuge vials, 1.5 mL *(InterScience Cat.# QS509GRDY or equivalent)*

25 Amino acid hydrolysis tubes (culture tubes, 6x50 mm) *(Waters Chromatography Division Cat.# WAT007571)*

2 reaction vials with 2 reaction vial caps *(Waters Chromatography Division Cat.# 7363)*

200 µL Microcapillary tips, polypropylene *(Sigma Chemical Co. Cat.# T-1906)*

30 Filter paper (0.45 µm or smaller) *(Millipore Corporation Cat.# HVLP04700 or equivalent)*

Acrodisc Filter, 0.2 µm *(Gelman Sciences Part# 4192)*

HPLC System

Beckman HPLC System containing the following modules: #507e autosampler, #166 programmable detector module, #126 programmable solvent module computer equipped with System Gold *Nouveau* software, CIN 2714 *(Beckman Instruments Inc.)*

5   Target vials *(Beckman Cat.# 887954, National Scientific Company Cat.# C4011-1)*

Target vial septa   *(Beckman Cat.# C40041, National Scientific Company Cat.# C4010-60)*

Target vial insert: low volume insert, 300 μL *(Beckman Cat.# C40042, National Scientific Company Cat.# C4010-630)*

10   Injection loops: 20 μL for Rheodyne # 7725 injector *(Beckman Cat.# 890870)*

Back-pressure unit, 100 psi *(Chromatographic Specialties Inc. Cat.# M100600130 or equivalent)*

Biorad column heater *(Biorad Cat.# 125-0425)*

Reversed-phase column: Pico-Tag column   *(Waters Cat.# WAT088131)*

15   Pre-column filter, 1/16", PEEK, 2 μm frit *(Supelco Cat.# Z-227323)*

Replacement filters for column pre-filter, 2 μm *(Sigma-Aldrich Cat.# Z22,733-1)*

Order of Events

Day 1 = Dry down samples and hydrolyse samples overnight.

20   Day 2 = Dry down standards, sample hydrolysates and derivatization blank.

Day 2 = Derivatize samples, standards and derivatization blank.

Use of Refrigerated Trap

If the trap has been used to dry down samples that contain HCl, exchange the trap for a clean trap before drying down samples containing 25   PITC (i.e., during the derivatization incubation, a clean trap should be installed).

Preparation of Amino Acid Stock Solutions

Preparation of 10 mM HCl:

Transfer 6N HCl from ampoule to an amino acid clean screw cap culture 30   tube.

Prepare 10 mM HCl by mixing 15 μL (6N HCl) + 8985 μL purified water.

Store in a screw cap culture tube at 2 - 8 C; expiry = 6 months

Preparation of Amino Acid Standard:

Amino acid stock solution #1: Transfer amino acid standard H (2.5 μmole/mL each amino acid) to an amino acid clean screw cap culture tube. Prepare amino acid stock solution #2 by mixing 100 μL amino acid standard

5   H (2.5 μmole/mL) + 400 μL 10 mM HCl and store in a screw cap culture tube. This represents 6 nmole of each amino acid/12 μL solution. (2.5 μmole/1000 μL)(100 μL) = 0.25 μmole 0.25 μmole/500 μL = 0.0005 μmole/μL) (0.0005 μmole/μL)(12 μL) = 0.006 μmole = 6 nmole).

Preparation of CMC Standards:

10   CMC stock solution #1: Dissolve 20 mg (catch weight) of CMC in 5.0 mL of 10 mM HCl to yield 0.0223 mmole CMC/mL. (Note: Adjust the volume of 10 mM HCl to the actual weight of CMC to yield 0.0223 mmole CMC/mL). 20 mg/179.2 = 0.112 mmole/5 mL = 0.0224 mmole/mL

CMC stock solution #2: Add 50 μL stock solution #1 to 450 μL of 10 mM

15   HCl. (0.0223 mmole/1000 μL) x 50 μL = 0.001115 mmole (0.001115 mmole/500 μL) x (10^6 nmole/mmole) = 2.23 nmole/μL

CMC stock solution #3: Add 70 μL stock solution #2 to 240 μL 10 mM HCl to yield 0.5 nmole CMC/μL. (70 μL)(2.23 nmole/μL) = 156 nmole; 156 nmole/310 μL = 0.5 nmole/μL

20   *Preparation of Dried Standard for Derivatization*

A volume of 20 μL reagent/sample is sufficient to derivatize 12.5 nmole of each amino acid or a total of 250 nmole amino acid [There is a total of 19 -amino groups x 12.5 nmole = 237.5 nmole total 250 nmole of amino acid]. Dispense 12 μL (6 nmole each amino acid) of standard solution #2 into

25   each of 4 amino acid clean hydrolysis tubes (6x50 mm). Dispense 12 μL of 10 mM HCl into an amino acid clean hydrolysis tube for the derivatization blank. Add 6 nmole of CMC to each of the standards and derivatization blank. Dry down samples on Pico-Tag station: Pressurize reaction vial with nitrogen. Close nitrogen valve. Open vacuum valve and allow to dry until

30   vacuum reaches approximately 65 mTorr. Close vacuum valve. Pressurize

reaction vial with nitrogen.

Sample Digestion

Dispense approximately 0.3 nmole of protein or approximately 10 nmole of a peptide into each of at least 4 amino acid clean hydrolysis tubes
5   (6x50 mm). This value is a starting point. If the composition is known, hydrolyse enough sample so that there is approximately 0.2 - 3.0 nmole each amino acid per 20 μL load. Once the samples have been analysed, the quantities loaded may have to be adjusted to obtain the suggested 0.2 - 3.0 range.

10   Target = 1-3 nmole most abundant amino acid/20 μL (as in standard curve). The derivatives will be dissolved in 120 μL and 20 μL will be loaded onto the column; Dilution = 120/20 = 6. Therefore, 6-18 nmole of the most abundant amino acid required. Most abundant AA: Target = (1-3 nmole most abundant AA/20 μL):

15   (# nmole most abundant AA)/(# nmole most abundant AA/nmole protein) = nmole protein required

(# nmole protein)(molecular weight of protein in ng/nmole)/(1000ng/μg) = μg protein required

(μg protein)/(concentration of protein in μg/μL) = μL protein required

20   Check the least abundant AA:

(# nmole protein)(# nmole least abundant AA/nmole protein) = nmole least abundant AA

(# nmole least abundant AA)(20/120) = nmole least abundant AA present (accounting for dilution of sample)

25   Ensure this value does not exceed the low limit (i.e., 0.2 nmole).

Determine the total number of nmole of amino acid being derivatized, i.e., 1 nmole of protein or peptide = x nmole of amino acid, where x = the number of residues.

For example, 1 nmole of hGH (191 amino acids) = 191 nmole amino acid
30   Therefore, 0.3 x 191 = 57.3 nmole amino acid < 250 nmole as stated above.

Place in reaction vial and dry down on Pico-Tag station as above. Add 200

μL 6N HCl to the bottom of the reaction vial. Add one crystal of phenol to the bottom of the reaction vial. Perform a total of 3 vacuum-nitrogen flush cycles [vacuum 1-2 torr and nitrogen 5 second flush].

5   Ensure that nitrogen and vacuum valves are closed. Place reaction vial on the Pico-Tag station. Open nitrogen valve for approximately 5 seconds; close nitrogen valve. Open vacuum valve until vacuum is approximately 1-2 torr (HCl should just start to evaporate); close vacuum valve. Repeat twice. Check bottom of reaction vial to ensure that there is still HCl present.

10   Place reaction vial into oven at 110 ± 1 C for 24 hours. After the hydrolysis time period, remove the reaction vial and allow to cool at room temperature (~10-15 minutes). Transfer hydrolysis tubes to a second reaction vial. Dry down samples on Pico-Tag station as above. Add 6 nmole of CMC per sample (i.e., 12 μL of CMC stock solution #3) and dry down samples on Pico-Tag station as in 6.4.4.

15   *Preparation of Redry/Derivatization Reagents*
In a 12x75 mm culture tube, prepare redrying reagent (methanol/water/TEA, 2:2:1) as follows: 100 μL methanol, 100 μL water, 50 μL TEA, 10 μL/sample = 25 samples.
In a 12x75 mm culture tube, prepare derivatization reagent
20   (methanol/TEA/water/PITC, 7:1:1:1) as follows: 210 μL methanol, 30 μL TEA, 30 μL water, 30 μL PITC, 20 μL/sample = 15 samples.

Derivatization of Standards and Samples
Redry step: Add 10 μL redrying reagent to each tube and mix. Dry down standards, samples and blank on Pico-Tag station as above.
25   Derivatization step: Add 20 μL derivatization reagent to each tube and mix. Allow to incubate at room temperature (20 - 25 C) for 20 min. Dry down standards, samples and blank on Pico-Tag station as above.

Preparation of HPLC Reagents
Solvent A (140 mM acetate, pH 6.4/ACN [94/6, v/v]):

11.5 g sodium acetate + 950 mL purified water + 500 μL TEA. Adjust pH to 6.4 with glacial acetic acid. Adjust volume to 1000 mL and filter. Dispense 893 mL buffer + 57 mL ACN.

Solvent B (60% ACN/water):

5   360 mL purified water + 540 mL ACN

Sample Diluent (5 mM Na_2HPO_4, pH 7.4/ACN [95/5, v/v]):

0.13 g $Na_2HPO_4 \cdot 7H_2O$ in 90 mL of purified water. Adjust to pH 7.4 with 5% H_3PO_4. 5% H_3PO_4 = 5.9 mL H_3PO_4 (85%) up to 100 mL with PW. Make final volume to 100 mL with PW and filter through 0.2 μm. Mix 95 mL of

10   the filtered buffer + 5.0 mL ACN using a stirrer.

20% MeOH (Storage solvent for unused lines):

Add 200 mL methanol to 800 mL purified water using a graduated glass cylinder.

Column Installation and Equilibration

15. Install column with PEEK tubing (0.010" I.D., blue) at the inlet and outlet and a 100 psi back pressure regulator at the detector outlet. Load reagents as follows:

TABLE 1

Reagent	Line
Solvent A (140 mM sodium acetate, pH 6.4/ACN [94/6, v/v])	A1 (Red)
Solvent B (60% ACN/purified water)	B1 (Red)
Purified water (degassed)	A2, B2 (Yellow)

Prime each line as per HPLC SOP# 84.030.0004.RR. Prime pumps with

20   wastevalve open, selector valve set at (A1, B1), and pumps set at 50% B [50% A]. Set the flow rate values onscreen to 3.000, 0.00 min. Wash the back side of the pistons of the HPLC pumps with water. Set the flow rate values to 0.000, 0.00 min. Close the drain valve by turning it clockwise until tight.

Set upper pump pressure limit to 2000 psi and lower pump pressure to

25   0.05 psi. Under DIRECT CONTROL, set pump to 100% B [0% A], (A1, B1).

With waste valve closed, set pump flow to 1 mL/min in 1 min. When pump has reached 1 mL/min, set 100% B [0% A] to 0% B [100% A] in 5 min. with selector valve at (A1, B1). After 5 min period, wash column with buffer A (to remove storage solvent) for 15 min. (at this point the baseline should be
5 stable).

HPLC Conditions
Experimental conditions:
Flow rate = 1.0 mL/min
Temperature = 38 C
10 Monitoring wavelength = 254 nm
Cooling tray = 6 C
Injection mode = Regular (Full loop requires a minimum of 100 μL sample.)

TABLE 2

Gradient table:

Time (min)	%A	%B	Duration (min)	Curve (CV)
INITIAL	100	0	-	-
0	58	46	10	3
10	0	100	0.5	0
10.5	0	100	5.5	0
16	100	0	1	0
17	100	0	8	0

15   Equilibration requires 20 mL of Eluent A and 5 mL of Solvent B: each run requires ~20 mL Solvent A and ~12 mL Solvent B.

Dissolution of Standards and Samples
        Dissolve each of the derivatized standards (6 nmole each amino acid) and each of the derivatized samples (1.6-16 nmole each amino acid) in 120 μL
20 sample diluent. Centrifuge each of the standards and samples at 13 000 rpm for 2 minutes. Load 20 μL of standard onto column, which is equivalent to 1

nmole each amino acid/20 µL load. Evaluate the standard curve, i.e., determine if all of the peaks have been identified as indicated in table 7.1. If all peaks can not be accounted for, prepare new buffers and re-run the standard curve. If peak identification is still a problem, then the HPLC may require

5   maintenance. Refer to the SOP entitled "Calibration Testing of the Beckman HPLCs". Load 20 µL of sample onto column, which is equivalent to 0.27 - 2.7 nmole each amino acid/20 µL load. 1.6/6 = 0.27 nmole each AA; 16/6 = 2.7 nmole each AA

Column Storage

10   Following completion of the shutdown method (see Exhibit 3), remove lines A1 and B1 prime lines with PW. Place both lines in 20% methanol/water and prime lines. Open waste valve. Before starting pumps, flush back side of pumps with PW. With selector valve set at 50% B [50% A], set the flow to 3.000 mL/min in 0.000 min and continue to flush the back side of the pumps

15   with up to 60 mL PW. Close waste valve.

Data Aanalysis

The elution order of the PTCaa is shown in the following table:

TABLE 3

Peak #	Amino acid	Peak #	Amino Acid
1	D (Asp)	10	P (Pro)
2	E (Glu)	11	Y (Tyr)
3	CMC	12	V (Val)
4	S (Ser)	13	M (Met) not used
5	G (Gly)	14	C (Cys) not used
6	H (His)	15	I (Ile)
7	R (Arg)	16	L (Leu)
8	T (Thr)	17	F (Phe)
9	A (Ala)	18	K (Lys)

Results

N-N conjugate: The amino acid composition of the conjugate matches the expected amino acid sequence of composition of GMCSF + RHAMM

5 indicating successful N-N conjugation.

TABLE 4

AA	GMCSF control			RHAMM^{N-N}GMCSF conjugate		
	Determined value	Integer value	Expected value	Determined value	Integer value	Expected value
G	3.02	3	3	3.10	3	3
H	6.03	6	6	2.93	3	3
R	8.28	8	8	5.78	6	6
V	5.87	6	6	4.95	5	5
F	4.97	5	5	5.18	5	5

K	6.83	-	7	7	6.05	6	6

C-N conjugate: The amino acid composition of single and double conjugate matches the expected composition of GMCSF + RHAMM and GMCSF + 2xRHAMM, respectively indicating successful C-N conjugation.

TABLE 5

AA	RHAMM^{C-N}GMCSF conjugate			RHAMM$_2$$^{C-N}$GMCSF conjugate		
	Determined value	Integer value	Expected value	Determined value	Integer value	Expected value
G	3.13	3	3	3.23	3	3
H	5.86	6	6	8.73	9	9
R	7.95	8	8	10.34	10	10
V	5.74	6	6	6.68	7	7
L	13.91	14	14	14.16	14	14
F	5.08	5	5	5.05	5	5
K	7.33	7	7	7.81	8	8

5 <u>Demonstration of Bioactivity of RHAMM-GM-CSF Conjugate:</u>

The following describes the procedures for an *in vitro* bioassay to determine the biological activity of LEUCOTROPIN™ (GM-CSF, Granulocyte-Macrophage Colony Stimulating Factor) using the TF-1 cell line and the MTT cell proliferation method. Materials required include the 10 following:

Materials:

TF-1 Cells: Human derived cell line of immature erythroid origin that proliferates dependently on GM-CSF.

Dulbecco's phosphate buffered saline (D-PBS): Gibco cat#. 14040-026 or 15 equivalent. Manufacturer's expiry date.

2.5M 2-mercaptoethanol (1mL): Mix 174μL 2-mercaptoethanol, ACS grade,

826μL D-PBS and sterile filter using a 3mL syringe and 0.22μm syringe filter.

Culture media: 500mL   DMEM, Gibco cat# 11965-050 or equivalent, 50mL Heat-Inactivated Fetal Bovine Serum (HI-FBS) from a certified and reliable source, 10μL 2.5M 2-mercaptoethanol

5   **Bovine serum albumin (BSA), fraction V,** Gibco cat# 11018-041 or equivalent
D-PBS + 0.1% BSA (sterile): 100mL D-PBS, 0.1g BSA, Dissolve BSA in D-PBS and sterile filter with 0.22μm filter.

4.0mg/mL MTT stock solution (sterile), 50mL: 0.2g MTT, Sigma cat# M5655 or equivalent, 50mL D-PBS, Dissolve the 0.2g of MTT in 50mL of D-PBS and

10 filter sterilize with 0.22μm filter. Store at 2-8°C, protect from light.

Assay media: 500mL of RPMI-1640 without phenol red, Gibco cat# 11835-030 or equivalent. 200μL of 25mg/mL insulin. 100mg sterile lyophilized insulin, Boehringer Mannheim cat#1 376 497 or equivalent. 2 D-PBS (adjusted to pH 3.0 with HCl, ACS grade or equivalent and sterile filtered)

15 Dissolve 100mg insulin (contents of one sterile lyophilized vial) in 4mL of sterile D-PBS (pH 3.0). Aliquot in 200μL aliquots and store at -10°C until needed. 200μL of 25mg/mL transferrin. 100mg lyophilized transferrin, Boehringer Mannheim cat# 1 073 974 or equivalent. D-PBS: Dissolve 100mg transferrin (contents of one sterile lyophilized vial) in 4mL D-PBS. Aliquot in

20 200μL aliquots and store at -10°C until needed.   20μL of 2.5M 2-mercaptoethanol. 1mL of 10mg/mL gentamicin, Gibco cat# 15710-023 or equivalent. Mix above (4.8.1 to 4.8.5), store at 2-8°C.

Cell Preparation

4×10^4 cells are needed/assay well, or 3.84×10^6 cells/96 well plate.

25 Remove the volume of cells needed as calculated in 6.1.2 and place them in a sterile centrifuge tube for centrifugation at 270xg for 5 minutes. Aspirate the supernatant and resuspend the cells in _ volume of culture media (4.4). Centrifuge cells again as per above. Aspirate the supernatant and resuspend cells in 30mL of culture media/96 well assay plate required (this will result in a

30 cell seeding concentration of 2×10^5 cells/mL). Transfer cell suspension to T-75 culture flask(s), 30mL/flask and incubate at 37°C, 8%CO_2 for 20-24 hours. On day zero of the bioassay, determine the viable cell count of the growth factor-deprived cells. Transfer the volume of cells needed for the assay (Section 6.2.2) to a sterile tube for centrifugation at 270xg for 5 minutes.

Aspirate the supernatant and resuspend the cell pellet in _ volume of fresh assay medium (4.8). Centrifuge the tube again. Aspirate the supernatant and resuspend the pellet in assay medium. The volume to be added is determined by the following equation: Volume of media (mL) = total # of cells / 4×10^5

5 cell/mL. 0.1mL of cell suspension is added/per well at a concentration of 4×10^5cells/mL resulting in 4×10^4 cells/well. The resuspended cells can be incubated after resuspension in the centrifuge tube at 37°C, 5% CO_2 for up to 6 hours until use. Dilute the 20μL of working stock standard (4.9, 10,000 IU/mL), 16.67x by adding 313.3μL of assay medium, giving a final volume of

10 333.3μL and a concentration of 600 IU/mL. Aliquot 100μL of the 600 IU/mL standard in triplicate to the first three wells designated for the standard curve on the plate. Add 50μL of assay medium to all the other wells designated for the standard curve on the plate. Using a 8-channel pipettor with 3 tips attached remove 50μL from the 100μL of 600IU/mL standard wells (6.4.2),

15 and transfer the volume to the next set of designated standard wells containing 50μL of assay medium. Aspirate well contents up and down five times to ensure proper mixing. This results in a two-fold dilution (i.e 600 IU/mL to 300 IU/mL). Remove 50μL from this dilution and transfer it to the next set of standard wells containing 50μL of assay media. Continue this until

20 the tenth dilution, where 50μL is then removed from the last set of standard wells and discarded. This results in equal volumes in all the standard wells.

Preparation of GM-CSF Test Sample

      Each test sample is prepared and diluted resulting in an approximate GM-CSF concentration of approximately 10ng/μL.

25 *Dilution of GM-CSF samples for Dose-Response Curves*

      Add 490μL of assay medium to the 10μL aliquot of 10ng/μL test sample from 6.5 to give a concentration of 200pg/μL. Remove 70μL of the 200pg/μL dilution and further dilute it to 40pg/μL by adding 280μL of assay medium. Aliquot 50μL of assay medium to all the wells to be used for the

30 sample dilutions, except for the first triplicate set. Aliquot 100μL of the 40pg/μL starting sample dilution in triplicate to the empty starting sample dilution wells. Transfer in triplicate, using a multichannel pipettor, 50μL of the starting dilution to the next set of triplicate wells containing 50μL of assay

medium. Aspirate well contents up and down five times to ensure proper mixing. Continue this 2-fold serial dilution until eight dilutions have been made. Remove 50μL from the last dilution so that they all have the same volume.

5   *Assay Procedures.*

Add 50μL of assay medium to 6 wells to act as blanks. Mix the cell suspension from above and add it to a sterile reservoir for dispensing. Add 100μL of the cell suspension (approximately 4×10^4 cells/100μL) to all plate wells containing 50μL of either standards, blanks or test samples, using the
10   multi-channel pipette. When adding cells be careful not to dip the pipette tips into the wells too far so as not to contact the standard/sample inside which would result in transfer of some of it to other standards/samples. Incubate the assay plate at 37°C, 5%CO_2 for 72 $\pm$ 5 hours.

Procedures for Day Three of the Bioassay
15   After incubation add 50μL of the 4mg/mL MTT solution to each well using an 8-channel pipette. Cover the plate with plate sealer and mix for approximately 30 seconds with the plate shaker. Remove plate sealer and incubate the plate for 3 $\pm$ 0.5 hours at 37°C, 5%CO_2. Remove plate and centrifuge at 1775xg for 15 minutes, with brake on high. Supernatant

20   *Removal Using Microplate Washer.*

Prior to aspirating assay plates check proper functioning of the plate washer by aspirating a blank plate filled with water only. Be sure that all 8 channels are aspirating efficiently and that none are plugged. Aspirate supernatant from all assay plates, using the microplate washer. Add
25   isopropanol, 50μL/well using a reagent reservoir and multi-channel pipette. Cover the plate with plate sealer and mix for 5 minutes using the plate shaker. Remove plate sealer and read the plates at 595nm using the microplate reader.

Results
30   n-b conjugate: mean potency is approximately 50% compared to native GMCSF.

TABLE 6

	Mean potency (IU/mg)	% potency compared to control
GMCSF control	1.24×10^7	100
RHAMM^{N-N}GMCSF conjugate	0.57×10^7	46

C-N conjugate: mean potency is approximately 50% compared to native GMCSF for a single conjugate, and approximately 40% compared to native GMCSF for a double conjugate.

5

TABLE 7

	Mean potency (IU/mg)	% potency compared to control
GMCSF control	1.23×10^7	100
RHAMM^{C-N}GMCSF conjugate	0.66×10^7	54
RHAMM$_2^{C-N}$GMCSF conjugate	0.48×10^7	39

EXAMPLE 17

Production of RHAMM-EPO direct conjugate using backbone engineered thioether linkage

Erythropoietin would benefit substantially from the distribution of HA in
10 bone marrow, as targeted by RHAMM peptides. Recombinant erythropoietin is currently produced in the glycosylated form, as the naked form is readily degraded and, therefore, therapeutically ineffective. Therefore, conjugation of non-glycosylated EPO to a RHAMM peptide will increase the half-life of EPO and direct the conjugate to tissues with a high concentration of HA. It has been reported that approximately
15 25% of HA is found in bone, which is an optimal target for EPO, and as such, in

addition to increasing half-life, conjugation to RHAMM may enhance target specificity.

Preparation of recombinant EPO

The complete coding DNA sequence of human erythropoietin was first
5   disclosed in US patent application Ser. No. 655.841, filed September 28, 1984, which was later issued as US Patent Number 4,703,008.

A iodoacetyl group must be added to the N-terminal of EPO for attachment of the RHAMM peptide. This may be accomplished by adjusting a EPO solution (originally in 10 mM phosphate, pH 6.5) to 60mM phosphate buffer and 0.15%
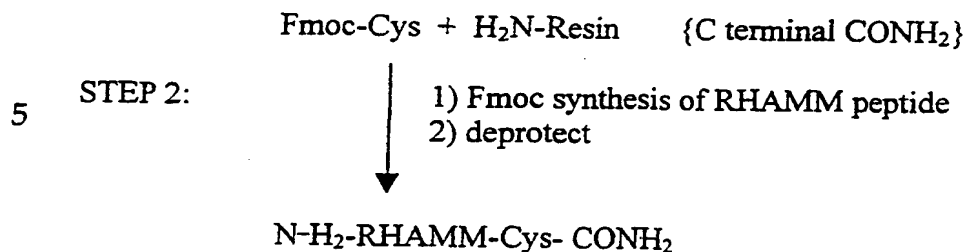10   pluronic at a pH of 6.0. Iodoacetic anhydride is added to a final 10-fold molar excess, i.e., an equimolar quantity is added in 10 aliqouts at 5 minute intervals at room temperature. Following iodoacetylation, the protein is purified by gel filtration on a PP10 coulumn. The preparation of recombinant EPO for thioether linkage conjugation to RHAMM is illustrated structurally below, where H2N- and –OH represent the N
15   and C terminals of EPO, respectively.

$$H_2N\text{--- EPO -(LysN--H}_2)\text{-OH}$$

STEP 1:   1) pH 6, 10 mM phosphate, 0.15% pluronic
                  2) iodoacetic anhydride

$$ICH_2C(=O)HN\text{--- EPO -(LysN--H}_2)\text{-OH}$$

20   Preparation of RHAMM peptide
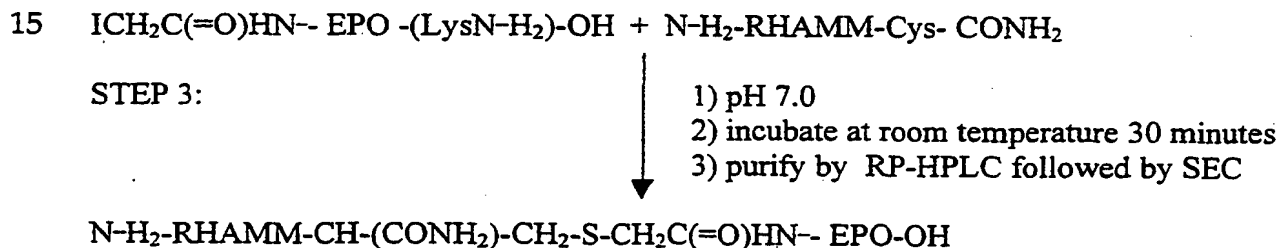
SEQ. I.D. 3 comprises the preferred sequence of RHAMM peptide for conjugation to EPO. The RHAMM peptide must be engineered with an C-terminal Cysteine group, which will later be ligated to the iodoacetylated N-terminal. To this end, a benzhydrylamine resin is utilized to produce a C-terminal amide and the first
25   residue coupled to the resin is the amino acid Cysteine. RHAMM is then

synthesized as per normal Boc or Fmoc solid phase peptide synthesis (SPPS) methods. Finally, SEC and/or RP-HPLC methods are used to purify the peptide. The process is illustrated structurally below.

Fmoc-Cys + H₂N-Resin   {C terminal CONH₂}

5   STEP 2:

1) Fmoc synthesis of RHAMM peptide
2) deprotect

N-H₂-RHAMM-Cys- CONH₂

Coupling of the RHAMM peptide and EPO

Iodoacetylated EPO is buffer exchanged into 10 mM phosphate, pH 7.0. The
10 RHAMM-Cys peptide is reconstituted in 10 mM phosphate, pH 7.0 and added to the Iodoacetylated EPO solution. The solution is allowed to incubate at room temperature for 30 minutes (reference if any). Purification of the conjugate is by performed by RP-HPLC followed by SEC. The structural representation of the conjugation reaction is illustrated below.

15   ICH₂C(=O)HN-- EPO -(LysN-H₂)-OH + N-H₂-RHAMM-Cys- CONH₂

STEP 3:

1) pH 7.0
2) incubate at room temperature 30 minutes
3) purify by RP-HPLC followed by SEC

N-H₂-RHAMM-CH-(CONH₂)-CH₂-S-CH₂C(=O)HN-- EPO-OH
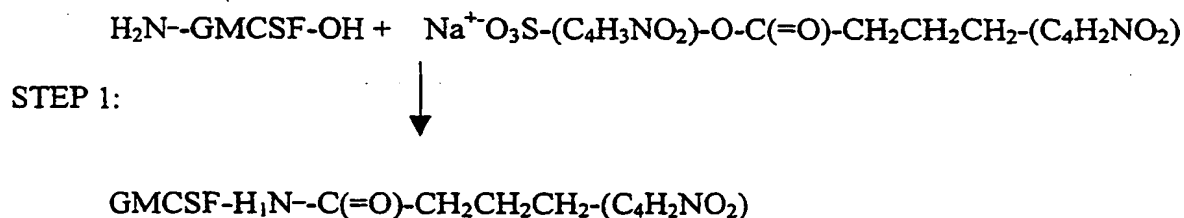
EXAMPLE 18

<u>Conjugation of RHAMM peptide and GMCSF by use of a hetrobifunctional cross-linker sulfo GMBS</u>

GM-CSF stimulates organ and tissue growth, enhancing amino acid uptake and
5 protein synthesis by while reducing protein catabolism. Conjugation of RHAMM to hGH would reduce dosing requirements and toxicity, while increasing target specificity by directing hGH to bone and cartilage.
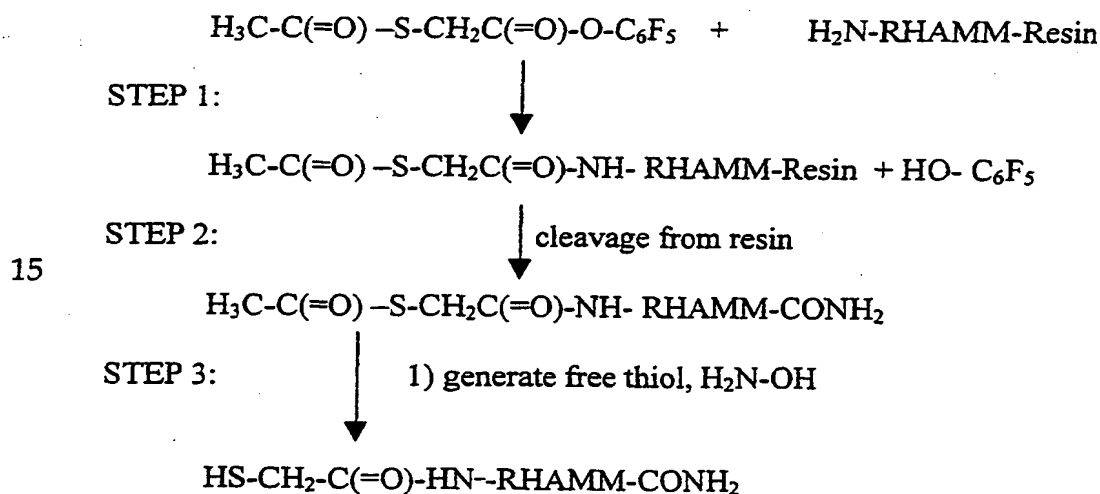
Preparation of recombinant GMCSF

The complete coding DNA sequence of human GM-CSF is given as GenBank
10 Accession No. E00951. Recombinant GM-CSF may be purchased from existing commercial sources, or produced by methods known to persons skilled in the art.

The protein is modified through the coupling of a heterobifunctional cross-linker, sulfo-GMBS, to the N-terminal via use of appropriate pH. This cross-linker contains an NHS ester at one end of the molecule and maleimide group at the other
15 end. In preparation of the modified protein, sulfo-GMBS cross-linker is dissolved in 50% acetonitrile and added to GMCSF at pH8 at a molar ratio of 1:3 (i.e., 1 linker :3 protein). Under these pH conditions, the NHS ester group reacts with the N-terminal of the protein. The unreacted maleimide group of the cross-linker is used in later coupling experiments to obtain the conjugate. The preparation of the modified protein
20 is illustrated structurally below.

$$H_2N\text{--GMCSF-OH} + Na^+O_3S\text{-}(C_4H_3NO_2)\text{-}O\text{-}C(=O)\text{-}CH_2CH_2CH_2\text{-}(C_4H_2NO_2)$$

STEP 1:

$$GMCSF\text{-}H_1N\text{--}C(=O)\text{-}CH_2CH_2CH_2\text{-}(C_4H_2NO_2)$$
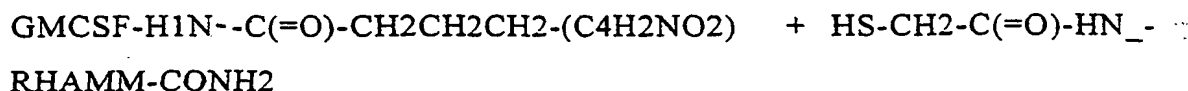
Preparation of RHAMM peptide

SEQ I.D. 3 is chosen as the RHAMM peptide and is synthesized with a N-terminal protected thiol moiety, which may be accomplished by standard SPPS methods using the following procedure. The RHAMM peptide is synthesized using
5 standard Fmoc SPPS protocols. The last residue to be added is a SAMA group to the RHAMM peptide, followed by cleavage from the resin. Prior to conjugation of the engineered RHAMM peptide with GMCSF, the free thiol on RHAMM must be generated by reaction with hydroxylamine. This is accomplished by dissolving the SAMA-RHAMM peptide in 20 mM phosphate, 150 mM NaCl, 10 mM EDTA at
10 pH 7, followed by addition of hydroxylamine (0.5 M in 20 mM phosphate, 150 mM NaCl, 25 mM EDTA). The preparation of the SAMA-RHAMM peptide and the generation of the free thiol group is illustrated structurally below.

$$H_3C\text{-}C(=O)\text{-}S\text{-}CH_2C(=O)\text{-}O\text{-}C_6F_5 \quad + \quad H_2N\text{-}RHAMM\text{-}Resin$$

STEP 1:

$$H_3C\text{-}C(=O)\text{-}S\text{-}CH_2C(=O)\text{-}NH\text{-}RHAMM\text{-}Resin \quad + \quad HO\text{-}C_6F_5$$

STEP 2:   cleavage from resin
15

$$H_3C\text{-}C(=O)\text{-}S\text{-}CH_2C(=O)\text{-}NH\text{-}RHAMM\text{-}CONH_2$$

STEP 3:   1) generate free thiol, $H_2N\text{-}OH$

$$HS\text{-}CH_2\text{-}C(=O)\text{-}HN\text{-}RHAMM\text{-}CONH_2$$
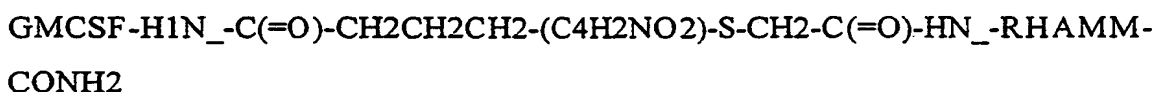
Coupling of RHAMM peptide and GMCSF

For the coupling of the RHAMM peptide to the N-terminal of the protein, conjugation should take place at a pH of 7 where the coupling occurs through the free thiol group of the N-terminal of the peptide and the unsaturated bond of the maleimide group on the protein (ref). The peptide is dissolved in 20 mM phosphate,

5   150 mM NaCl, 10 mM EDTA and the protein was buffer exchanged into 20 mM phosphate, 150 mM NaCl, pH7 after the modification reaction. The solution conditions are as follows: incubated at 0°C for 30 minutes and at room temperature for 45 minutes (ref). The final product is lyophilized and purified by RP-HPLC using water/TFA and acetonitrile/TFA. The product is eluted by buffer exchange into an

10 appropriate buffer using SEC. The coupling reaction between GMCSF and prepared RHAMM peptide is illustrated structurally below.

GMCSF-H1N--C(=O)-CH2CH2CH2-(C4H2NO2)   +   HS-CH2-C(=O)-HN_- RHAMM-CONH2

15 1) pH 7
    2) 20 mM phosphate, 150 mM NaCl
    3) EDTA

GMCSF-H1N_-C(=O)-CH2CH2CH2-(C4H2NO2)-S-CH2-C(=O)-HN_-RHAMM-CONH2

20 *Diagramatic representation of reaction steps*

Heterobifunctional linker reactions:

1. Modify protein with sulfo-GMBS:

2. Synthesize peptide with SAMA N-terminal:

3. Deprotect peptide with hydroxylamine:

4. Link modified protein to deprotected peptide:

5 EXAMPLE 19

Conjugation of RHAMM peptide and hGH by use of a hetrobifunctional cross-linker sulfo GMBS

Human growth hormone stimulates organ and tissue growth, enhancing amino acid uptake and protein synthesis by while reducing protein catabolism. Conjugation
10 of RHAMM to hGH would reduce dosing requirements and toxicity, while increasing target specificity by directing hGH to bone and cartilage.

Preparation of recombinant hGH

The complete coding sequence of human growth hormone is given in For this

method of coupling, the protein does not require modification or engineering prior to the conjugation step. Recombinant hGH may be purchased from existing commercial sources, or produced by methods known to persons skilled in the art.

The protein is modified through the coupling of a heterobifunctional cross-
5 linker, sulfo-GMBS, to the N-terminal via use of appropriate pH. This cross-linker contains an NHS ester at one end of the molecule and maleimide group at the other end. In preparation of the modified protein, sulfo-GMBS cross-linker is dissolved in 50% ace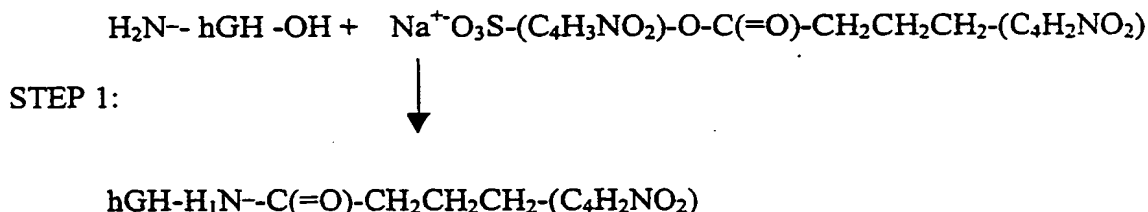tonitrile and added to hGH at pH8 at a molar ratio of 1:3 (i.e., 1 linker :3 protein). Under these pH conditions, the NHS ester group reacts with the N-terminal
10 of the protein. The unreacted maleimide group of the cross-linker is used in later coupling experiments to obtain the conjugate. The preparation of the modified protein is illustrated structurally below.

$$H_2N\text{--} hGH \text{-OH} + \quad Na^+O_3S\text{-}(C_4H_3NO_2)\text{-}O\text{-}C(=O)\text{-}CH_2CH_2CH_2\text{-}(C_4H_2NO_2)$$

STEP 1:

$$hGH\text{-}H_1N\text{--}C(=O)\text{-}CH_2CH_2CH_2\text{-}(C_4H_2NO_2)$$

15

Preparation of RHAMM peptide

SEQ I.D. 3 is chosen as the RHAMM peptide and is synthesized with a N-terminal protected thiol moiety, which may be accomplished by standard SPPS methods using the following procedure. The RHAMM peptide is synthesized using
20 standard Fmoc SPPS protocols. The last residue to be added is a SAMA group to the RHAMM peptide, followed by cleavage from the resin. Prior to conjugation of the engineered RHAMM peptide with hGH, the free thiol on RHAMM must be generated by reaction with hydroxylamine. This is accomplished by dissolving the SAMA-RHAMM peptide in 20 mM phosphate, 150 mM NaCl, 10 mM EDTA at
25 pH 7, followed by addition of hydroxylamine (0.5 M in 20 mM phosphate, 150 mM

NaCl, 25 mM EDTA). The preparation of the SAMA-RHAMM peptide and the generation of the free thiol group is illustrated structurally below.

$$H_3C\text{-}C(=O)\text{-}S\text{-}CH_2C(=O)\text{-}O\text{-}C_6F_5 \quad + \quad H_2N\text{-}RHAMM\text{-}Resin$$

STEP 1:

$$H_3C\text{-}C(=O)\text{-}S\text{-}CH_2C(=O)\text{-}NH\text{-}RHAMM\text{-}Resin \ + HO\text{-}C_6F_5$$

5

STEP 2: | cleavage from resin

$$H_3C\text{-}C(=O)\text{-}S\text{-}CH_2C(=O)\text{-}NH\text{-}RHAMM\text{-}CONH_2$$

STEP 3: | 1) generate free thiol, $H_2N\text{-}OH$

$$HS\text{-}CH_2\text{-}C(=O)\text{-}HN\text{-}RHAMM\text{-}CONH_2$$

10

Coupling of RHAMM peptide and hGH

For the coupling of the RHAMM peptide to the N-terminal of the protein, conjugation should take place at a pH of 7 where the coupling occurs through the free thiol group of the N-terminal of the peptide and the unsaturated bond of the

15 maleimide group on the protein (ref). The peptide is dissolved in 20 mM phosphate, 150 mM NaCl, 10 mM EDTA and the protein was buffer exchanged into 20 mM phosphate, 150 mM NaCl, pH7 after the modification reaction. The solution conditions are as follows: incubated at 0°C for 30 minutes and at room temperature for 45 minutes (ref). The final product is lyophilized and purified by RP-HPLC using

20 water/TFA and acetonitrile/TFA. The product is eluted by buffer exchange into an appropriate buffer using SEC. The coupling reaction between hGH and prepared

RHAMM peptide is illustrated structurally below.

$$hGH \text{ -}H_1N\text{--}C(=O)\text{-}CH_2CH_2CH_2\text{-}(C_4H_2NO_2) + HS\text{-}CH_2\text{-}C(=O)\text{-}HN\text{--}RHAMM\text{-}CONH_2$$

1) pH 7
2) 20 mM phosphate, 150 mM NaCl
3) EDTA

5

$$hGH \text{ -}H_1N\text{--}C(=O)\text{-}CH_2CH_2CH_2\text{-}(C_4H_2NO_2)\text{-}S\text{-}CH_2\text{-}C(=O)\text{-}HN\text{--}RHAMM\text{-}CONH_2$$

EXAMPLE 20

10 Conjugation of PEG-RHAMM peptide to EPO by use of a heterobifunctional cross-linker sulfo GMBS

Preparation of recombinant EPO

The complete coding DNA sequence of human erythropoietin was first disclosed in US patent application Ser. No. 655.841, filed September 28, 1984, which
15 was later issued as US Patent Number 4,703,008. Recombinant EPO can be produced by methods known to persons skilled in the art, or purchased from existing commercial sources. For this method of coupling, the protein requires modification or engineering prior to the conjugation step.

The protein is modified through the coupling of a heterobifunctional cross-
20 linker, sulfo-GMBS, to the N-terminal via use of appropriate pH. This cross-linker

contains an NHS ester at one end of the molecule and maleimide group at the other end. In preparation of the modified protein, sulfo-GMBS cross-linker is dissolved in 50% acetonitrile and added to EPO at pH8 at a molar ratio of 1:3 (i.e., 1 linker :3 protein). Under these pH conditions, the NHS ester group reacts with the N-terminal

5   of the protein. The unreacted maleimide group of the cross-linker is used in later coupling experiments to obtain the conjugate. The preparation of the modified protein is illustrated structurally below.
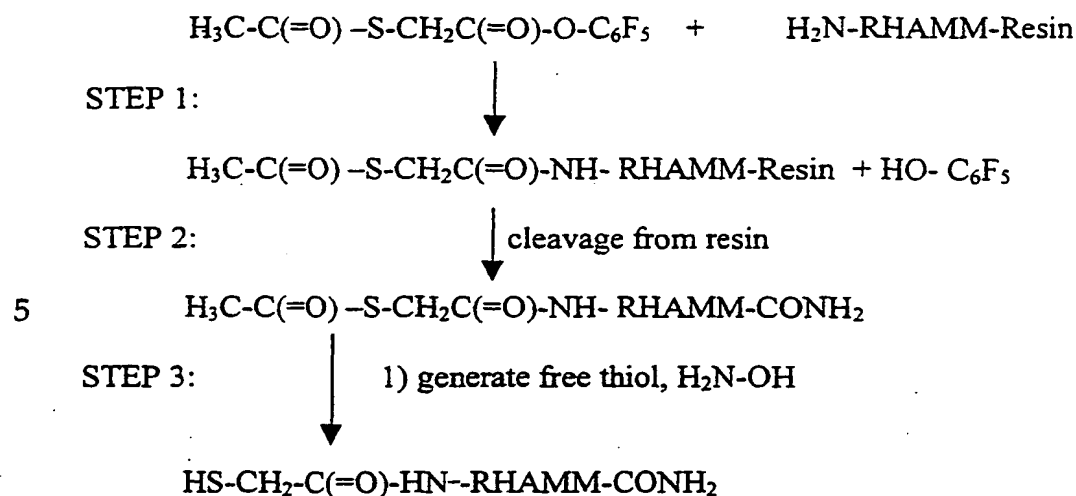
$$H_2N\text{--EPO-OH} + \quad Na^+\text{-}O_3S\text{-}(C_4H_3NO_2)\text{-}O\text{-}C(=O)\text{-}CH_2CH_2CH_2\text{-}(C_4H_2NO_2)$$

STEP 1:
$$\downarrow$$

$$EPO\text{-}H_1N\text{--}C(=O)\text{-}CH_2CH_2CH_2\text{-}(C_4H_2NO_2)$$

10

Preparation of RHAMM peptide

SEQ I.D. 3 is chosen as the RHAMM peptide and is synthesized with a N-terminal protected thiol moiety, which may be accomplished by standard SPPS methods using the following procedure. The RHAMM peptide is synthesized using

15   standard Fmoc SPPS protocols. The last residue to be added is a SAMA group to the RHAMM peptide, followed by cleavage from the resin. Prior to conjugation of the engineered RHAMM peptide with EPO, the free thiol on RHAMM must be generated by reaction with hydroxylamine. This is accomplished by dissolving the SAMA-RHAMM peptide in 20 mM phosphate, 150 mM NaCl, 10 mM EDTA at

20   pH 7, followed by addition of hydroxylamine (0.5 M in 20 mM phosphate, 150 mM NaCl, 25 mM EDTA). The preparation of the SAMA-RHAMM peptide and the generation of the free thiol group is illustrated structurally below.

$$H_3C\text{-}C(=O)\,\text{-}S\text{-}CH_2C(=O)\text{-}O\text{-}C_6F_5 \quad + \quad H_2N\text{-RHAMM-Resin}$$

STEP 1:

$$H_3C\text{-}C(=O)\,\text{-}S\text{-}CH_2C(=O)\text{-}NH\text{- RHAMM-Resin} \;+\; HO\text{- }C_6F_5$$

STEP 2:   cleavage from resin

5          $$H_3C\text{-}C(=O)\,\text{-}S\text{-}CH_2C(=O)\text{-}NH\text{- RHAMM-CONH}_2$$

STEP 3:   1) generate free thiol, $H_2N\text{-OH}$

$$HS\text{-}CH_2\text{-}C(=O)\text{-}HN\text{--RHAMM-CONH}_2$$

Coupling of RHAMM peptide and EPO

10   For the coupling of the RHAMM peptide to the N-terminal of the protein, conjugation should take place at a pH of 7 where the coupling occurs through the free thiol group of the N-terminal of the peptide and the unsaturated bond of the maleimide group on the protein (ref). The peptide is dissolved in 20 mM phosphate, 150 mM NaCl, 10 mM EDTA and the protein was buffer exchanged into 20 mM

15 phosphate, 150 mM NaCl, pH7 after the modification reaction. The solution conditions are as follows: incubated at 0°C for 30 minutes and at room temperature for 45 minutes (ref). The final product is lyophilized and purified by RP-HPLC using water/TFA and acetonitrile/TFA. The product is eluted by buffer exchange into an appropriate buffer using SEC. The coupling reaction between EPO and prepared

20 RHAMM peptide is illustrated structurally below.

EPO-H₁N--C(=O)-CH₂CH₂CH₂-(C₄H₂NO₂) + HS-CH₂-C(=O)-HN--RHAMM-CONH₂

1) pH 7
2) 20 mM phosphate, 150 mM NaCl
3) EDTA

EPO-H₁N--C(=O)-CH₂CH₂CH₂-(C₄H₂NO₂)-S-CH₂-C(=O)-HN--RHAMM-CONH₂

EXAMPLE 21

Conjugation of RHAMM peptide to GMCSF by backbone engineered thioether linkage using a bifunctional PEG linkage
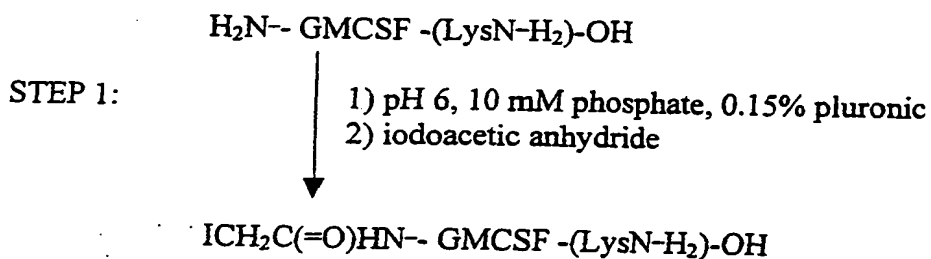
GMCSF would benefit substantially from the distribution of HA in bone

10 marrow, as targeted by RHAMM peptides. Recombinant erythropoietin is currently produced in the glycosylated form, as the naked form is readily degraded and, therefore, therapeutically ineffective. Therefore, conjugation of non-glycosylated GM-CSF to a RHAMM peptide will increase the half-life of GM-CSF and direct the conjugate to tissues with a high concentration of HA. It has been reported that

15 approximately 25% of HA is found in bone, which is an optimal target for GM-CSF, and as such, in addition to increasing half-life, conjugation to RHAMM may enhance target specificity.

Preparation of recombinant GMCSF

The complete coding DNA sequence of human GM-CSF is given as GenBank

20 Accession No. E00951. Recombinant GM-CSF may be purchased from existing commercial sources, or produced by methods known to persons skilled in the art.

A iodoacetyl group must be added to the N-terminal of GMCSF for attachment of the RHAMM peptide. This may be accomplished by adjusting a GMCSF solution (originally in 10 mM phosphate, pH 6.5) to 60mM phosphate

buffer and 0.15% pluronic at a pH of 6.0. Iodoacetic anhydride is added to a final 10-fold molar excess, i.e., an equimolar quantity is added in 10 aliqouts at 5 minute intervals at room temperature (ref). Following iodoacetylation, the protein is purified by gel filtration on a PP10 coulumn. The preparation of recombinant GMCSF for

10 thioether linkage conjugation to RHAMM is illustrated structurally below, where H2N- and –OH represent the N and C terminals of GMCSF, respectively.

$$H_2N\text{--} GMCSF \text{-}(LysN\text{--}H_2)\text{-}OH$$

STEP 1:

1) pH 6, 10 mM phosphate, 0.15% pluronic
2) iodoacetic anhydride

$$ICH_2C(=O)HN\text{--} GMCSF \text{-}(LysN\text{--}H_2)\text{-}OH$$

15

Preparation of C-terminal PEGylated RHAMM peptide

SEQ. I.D. 3 comprises the preferred sequence of RHAMM peptide for conjugation to PEG and hGH. The RHAMM peptide must be engineered with a PEG linker molecule and a Cys residue at the C-terminal end of RHAMM, which will later be

20 ligated to the iodoacetylated N-terminal of GMCSF. To this end, a benzhydrylamine resin is utilized to produce a C-terminal amide and the first residue coupled to the resin is the amino acid Cysteine. A bifunctional PEG molecule is then coupled to the Cys residue, followed by RHAMM synthesis as per normal Fmoc SPPS methods (Boc methods are not recommended for this procedure (Lu and Felix, 1993)). Finally,

25 SEC and/or RP-HPLC methods are used to purify the peptide. The process is illustrated structurally below.

Fmoc -Cys + H₂N-Resin   {C terminal CONH₂}

STEP 2: | 1) coupling
                     | 2) deprotect
                     ▼

N-H₂- Cys-Resin

STEP 2: | 1) PEG addition (Fmoc-NH-PEG-CO-NHS)
                     | 2)
                     ▼

5   Fmoc-NH-PEG-CONH-Cys-Resin

STEP 3: | 1) deprotect
                     | 2) Fmoc synthesis of RHAMM peptide
                     ▼

N-H₂-RHAMM-CONH-PEG-CONH-Cys-CONH₂

Coupling of RHAMM-PEG to GMCSF

Iodoacetylated GMCSF is buffer exchanged into 10 mM phosphate, pH7.
The PEGylated RHAMM-Cys peptide is reconstituted in 10 mM phosphate, pH7
and added to the iodoacetylated GMCSF solution. The solution is allowed to
15 incubate at room temperature for 30 minutes (ref). Purification of the conjugate is by
AEX followed by SEC. The structural representation of the conjugation reaction is
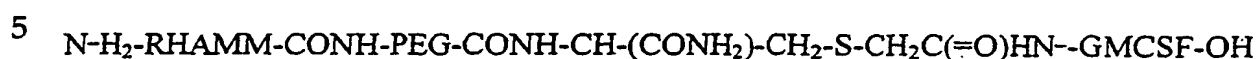
illustrated below.

ICH₂C(=O)HN--GMCSF -OH + N-H₂-RHAMM-CONH-PEG-CONH-Cys-CONH₂

STEP 4:
　　　　　　　　　　　　　　　1) pH 7
　　　　　　　　　　　　　　　2) incubate at room temperature 30 minutes
　　　　　　　　　　　　　　　3) purify by AEXfollowed by SEC

5　N-H₂-RHAMM-CONH-PEG-CONH-CH-(CONH₂)-CH₂-S-CH₂C(=O)HN--GMCSF-OH

EXAMPLE 22

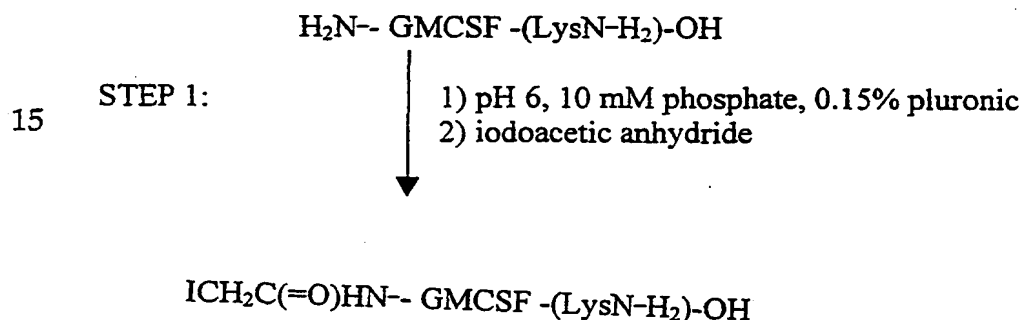10　Production of RHAMM-GMCSF direct conjugate using backbone engineered thioether linkage: alternate method

GMCSF would benefit substantially from the distribution of HA in bone marrow, as targeted by RHAMM peptides. Recombinant erythropoietin is currently produced in the glycosylated form, as the naked form is readily degraded and,

15　therefore, therapeutically ineffective. Therefore, conjugation of non-glycosylated GM-CSF to a RHAMM peptide will increase the half-life of GM-CSF and direct the conjugate to tissues with a high concentration of HA. It has been reported that approximately 25% of HA is found in bone, which is an optimal target for GM-CSF, and as such, in addition to increasing half-life, conjugation to RHAMM may enhance

20　target specificity.

Preparation of recombinant GMCSF

The complete coding DNA sequence of human GM-CSF is given as GenBank

Accession No. E00951. Recombinant GM-CSF may be purchased from existing commercial sources, or produced by methods known to persons skilled in the art.
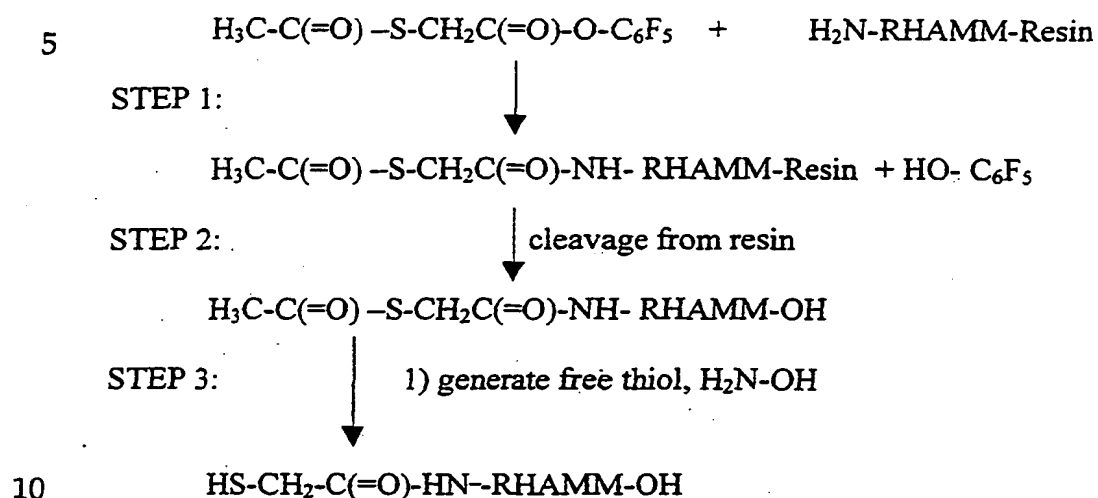
5   A iodoacetyl group must be added to the N-terminal of GMCSF for attachment of the RHAMM peptide. This may be accomplished by adjusting a GMCSF solution (originally in 10 mM phosphate, pH 6.5) to 60mM phosphate buffer and 0.15% pluronic at a pH of 6.0. Iodoacetic anhydride is added to a final 10-fold molar excess, i.e., an equimolar quantity is added in 10 aliqouts at 5 minute

10 intervals at room temperature (ref). Following iodoacetylation, the protein is purified by gel filtration on a PP10 coulumn. The preparation of recombinant GMCSF for thioether linkage conjugation to RHAMM is illustrated structurally below, where H2N- and –OH represent the N and C terminals of GMCSF, respectively.

$$H_2N\text{-- GMCSF -(LysN-H}_2)\text{-OH}$$

15   STEP 1:   1) pH 6, 10 mM phosphate, 0.15% pluronic
                              2) iodoacetic anhydride

$$ICH_2C(=O)HN\text{-- GMCSF -(LysN-H}_2)\text{-OH}$$

Preparation of RHAMM peptide

       SEQ I.D. 3 is chosen as the RHAMM peptide and is synthesized with a N-

20 terminal protected thiol moiety, which may be accomplished by standard SPPS methods using the following procedure. The RHAMM peptide is synthesized using standard Fmoc SPPS protocols. The last residue to be added is a SAMA group to the RHAMM peptide, followed by cleavage from the resin. Prior to conjugation of the engineered RHAMM peptide with GMCSF, the free thiol on RHAMM must be

25 generated by reaction with hydroxylamine. This is accomplished by dissolving the SAMA-RHAMM peptide in 20 mM phosphate, 150 mM NaCl, 10 mM EDTA at

pH 7, followed by addition of hydroxylamine (0.5 M in 20 mM phosphate, 150 mM NaCl, 25 mM EDTA). The preparation of the SAMA-RHAMM peptide and the generation of the free thiol group is illustrated structurally below.

5

$H_3C-C(=O)-S-CH_2C(=O)-O-C_6F_5$ + $H_2N-RHAMM-Resin$

STEP 1:

$H_3C-C(=O)-S-CH_2C(=O)-NH- RHAMM-Resin$ + HO- C_6F_5

STEP 2: | cleavage from resin

$H_3C-C(=O)-S-CH_2C(=O)-NH- RHAMM-OH$

STEP 3:   1) generate free thiol, H_2N-OH

10        $HS-CH_2-C(=O)-HN-RHAMM-OH$

Coupling of the RHAMM peptide and GMCSF

Iodoacetylated GMCSF is buffer exchanged into 10 mM phosphate, pH 7.0.
15 The SAMA-RHAMM peptide is reconstituted in 10 mM phosphate, pH 7.0 and added to the Iodoacetylated GMCSF solution. The solution is allowed to incubate at room temperature for 30 minutes (reference if any). Purification of the conjugate is by performed by RP-HPLC followed by SEC. The structural representation of the conjugation reaction is illustrated below.
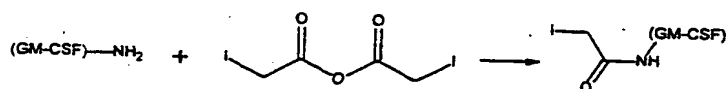
ICH$_2$C(=O)HN-- GMCSF -OH + HS-CH$_2$-C(=O)- HN- -RHAMM-CONH$_2$

STEP 3:

1) pH 7.0
2) incubate at room temperature 30 minutes
3) purify by RP-HPLC followed by SEC

(CONH$_2$)- RHAMM-N-HC(=O)CH$_2$-S-CH$_2$C(=O)HN-- GMCSF-OH
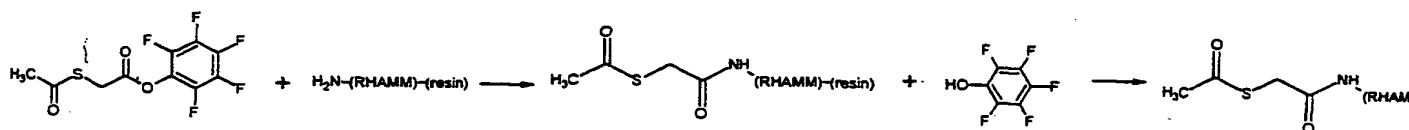
5 *Diagramatic representation of reaction steps*
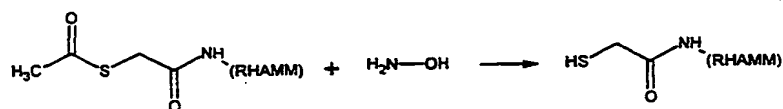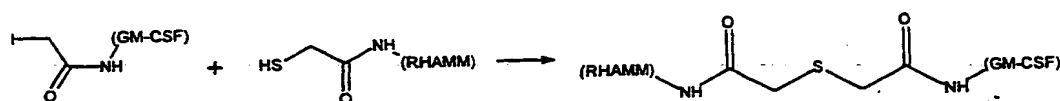
1. Iodoacetylate protein:

2. Synthesize peptide with SAMA N-terminal:

3. Deprotect peptide with hydroxylamine:

10 4. Couple modified protein to the free thiol of the peptide:

Having illustrated this alternate method for the production of RHAMM-GMCSF

direct conjugate using backbone engineered thioether linkage, it should be apparent to a person skilled in the art that this alternate method may be readily modified to conjugate RHAMM to other proteins.
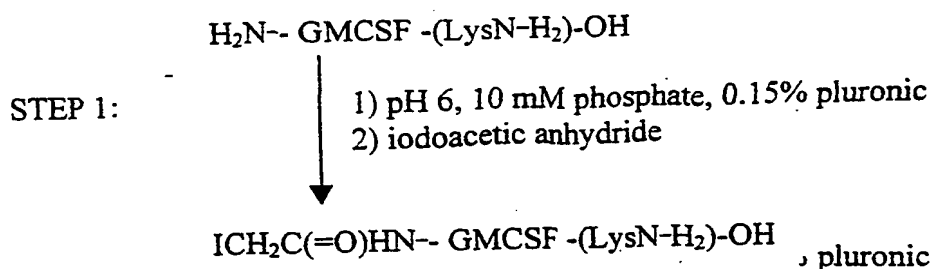

5 **EXAMPLE 22**

Production of RHAMM-GMCSF direct conjugate using backbone engineered thioether linkage: alternate method

GMCSF would benefit substantially from the distribution of HA in bone marrow, as targeted by RHAMM peptides. Recombinant erythropoietin is currently 10 produced in the glycosylated form, as the naked form is readily degraded and, therefore, therapeutically ineffective. Therefore, conjugation of non-glycosylated GM-CSF to a RHAMM peptide will increase the half-life of GM-CSF and direct the conjugate to tissues with a high concentration of HA. It has been reported that approximately 25% of HA is found in bone, which is an optimal target for GM-CSF, 15 and as such, in addition to increasing half-life, conjugation to RHAMM may enhance target specificity.

Preparation of recombinant GMCSF

The complete coding DNA sequence of human GM-CSF is given as GenBank Accession No. E00951. Recombinant GM-CSF may be purchased from existing 20 commercial sources, or produced by methods known to persons skilled in the art.

A iodoacetyl group must be added to the N-terminal of GMCSF for attachment of the RHAMM peptide. This may be accomplished by adjusting a GMCSF solution (originally in 10 mM phosphate, pH 6.5) to 60mM phosphate buffer and 0.15% pluronic at a pH of 6.0. Iodoacetic anhydride is added to a final 10-25 fold molar excess, i.e., an equimolar quantity is added in 10 aliqouts at 5 minute intervals at room temperature (ref). Following iodoacetylation, the protein is purified by gel filtration on a PP10 coulumn. The preparation of recombinant GMCSF for thioether linkage conjugation to RHAMM is illustrated structurally below, where

H$_2$N-- GMCSF -(LysN-H$_2$)-OH

STEP 1:

    1) pH 6, 10 mM phosphate, 0.15% pluronic
    2) iodoacetic anhydride

ICH$_2$C(=O)HN-- GMCSF -(LysN-H$_2$)-OH , pluronic

5

Preparation of RHAMM peptide

SEQ I.D. 3 is chosen as the RHAMM peptide and is synthesized with a N-terminal protected thiol moiety, which may be accomplished by standard SPPS methods using the following procedure. The RHAMM peptide is synthesized using

10 standard Fmoc SPPS protocols. The last residue to be added is a SAMA group to the RHAMM peptide, followed by cleavage from the resin. Prior to conjugation of the engineered RHAMM peptide with GMCSF, the free thiol on RHAMM must be generated by reaction with hydroxylamine. This is accomplished by dissolving the SAMA-RHAMM peptide in 20 mM phosphate, 150 mM NaCl, 10 mM EDTA at

15 pH 7, followed by addition of hydroxylamine (0.5 M in 20 mM phosphate, 150 mM NaCl, 25 mM EDTA). The preparation of the SAMA-RHAMM peptide and the generation of the free thiol group is illustrated structurally below.

H$_3$C-C(=O) –S-CH$_2$C(=O)-O-C$_6$F$_5$ +   H$_2$N-RHAMM-Resin

STEP 1:

H$_3$C-C(=O) –S-CH$_2$C(=O)-NH- RHAMM-Resin + HO- C$_6$F$_5$

STEP 2:   cleavage from resin

20   H$_3$C-C(=O) –S-CH$_2$C(=O)-NH- RHAMM-OH

STEP 3:   1) generate free thiol, H$_2$N-OH

HS-CH$_2$-C(=O)-HN--RHAMM-OH

Coupling of the RHAMM peptide and GMCSF

5   Iodoacetylated GMCSF is buffer exchanged into 10 mM phosphate, pH 7.0. The SAMA-RHAMM peptide is reconstituted in 10 mM phosphate, pH 7.0 and added to the Iodoacetylated GMCSF solution. The solution is allowed to incubate at room temperature for 30 minutes (reference if any). Purification of the conjugate is by performed by RP-HPLC followed by SEC. The structural representation of the

10 conjugation reaction is illustrated below.
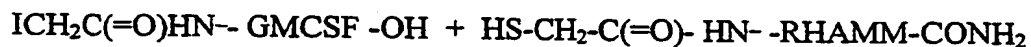
$ICH_2C(=O)HN-- GMCSF -OH + HS-CH_2-C(=O)- HN- -RHAMM-CONH_2$

STEP 3:

                       1) pH 7.0
                       2) incubate at room temperature 30 minutes
                       3) purify by RP-HPLC followed by SEC

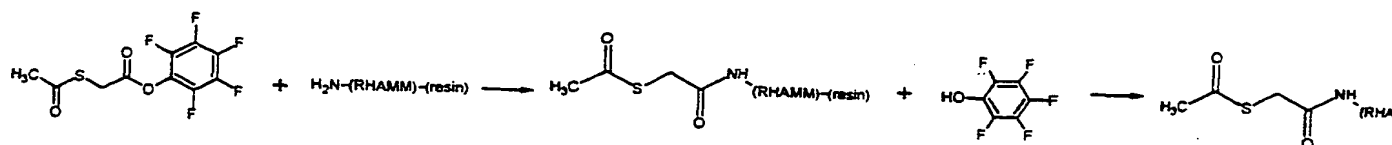$(CONH_2)- RHAMM-N-HC(=O)CH_2-S-CH_2C(=O)HN-- GMCSF-OH$

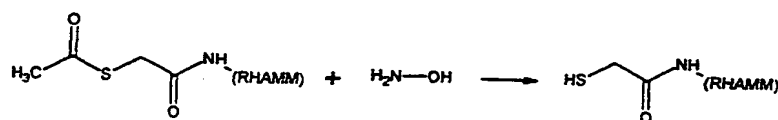*Diagramatic representation of reaction steps*
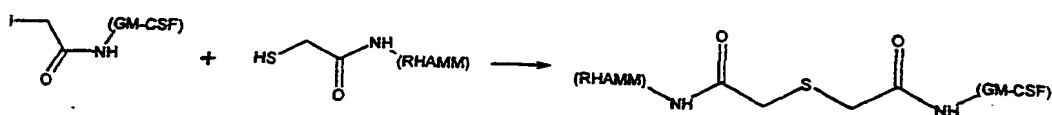
15

1. Iodoacetylate protein:
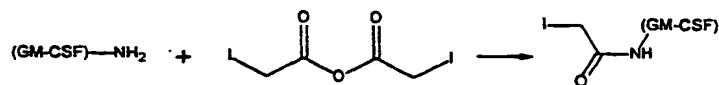
2. Synthesize peptide with SAMA N-terminal:

3. Deprotect peptide with hydroxylamine:

4. Couple modified protein to the free thiol of the peptide:

5   Having illustrated this alternate method for the production of RHAMM-GMCSF direct conjugate using backbone engineered thioether linkage, it should be apparent to a person skilled in the art that this alternate method may be readily modified to conjugate RHAMM to other proteins.

All publications, patents and patent applications referred to herein are
10   incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated by those skilled in the art
15   that invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

References

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WE CLAM:

1.   A conjugate comprising an HA-binding protein contiguous with, or coupled to a polypeptide conjugated to a therapeutic agent.

2.   A conjugate comprising an HA-binding peptide conjugated to a therapeutic agent.

3.   An isolated and purified nucleic acid sequence encoding an HA-binding protein or peptide in sequence with a therapeutic agent.

4.   An isolated and purified nucleic acid sequence according to claim 3 wherein the nucleic acid sequence does not encode an intervening amino acid sequence between the HA-binding peptide in sequence and the therapeutic agent.

5.   A conjugate according to any one of claims 1-4 wherein the HA-binding protein is a receptor for hyaluronan-mediated motility (RHAMM), or a variant thereof capable of binding HA, or an anti-HA antibody or variant thereof capable of binding HA.

6.   A conjugate according to claim 5 wherein the HA-binding protein is a polypeptide comprising an amino acid sequence of SEQ ID NO. 1, SEQ ID NO. 2 or SEQ ID NO. 3, or a fragment, analog or derivative thereof which maintains the ability to bind HA.

7.   A conjugate according to claim 5 wherein the HA-binding protein has an amino acid sequence comprising a structure of B^1 - A_n - B^2 wherein B^1 and B^2 are the same or different basic amino acid residues and A_n is an amino acid sequence containing seven or eight amino acid resides which are the same or different and are neutral or basic amino acids.

8.   A conjugate according to claim 7 wherein the HA-binding peptide comprises an amino acid sequence selected from the group consisting of KQKKHXXK, KIKHXXKUK, KLRsQLXKIR and KLRSQLXKRK wherein

each X is independently selected from V or D

9.   A conjugate according to claim 8 wherein the HA-binding peptide comprises an amino acid sequence according to one of SEQ. ID. NO.: 7 , SEQ. ID. NO.:8, SEQ. ID. NO.: 9, or SEQ. ID. NO. 10.

10.   A conjugate according to claim 5 wherein the HA binding peptide has an amino acid sequence comprising the following Formula I:

$X_1 - X_2 - X_1 - X_3 - X_4 - X_3 - X_4 - X_3 - X_3 - X_3 - X_5 - X_6 - X_6 - X_6 - X_1$

wherein:

each X_1 is independently selected from a hydroxy amino acid residue;

each X_2 is independently selected from a sulfur containing amino acid residue;

each X_3 is independently selected from a basic amino acid residue;

each X_4 is independently selected from an imino or aromatic amino acid residue;

each X_5 is independently selected from a dicarboxylic acid amino acid residue; and

each X_6 is independently selected from an aliphatic amino acid residue; and preferably:

each X_1 is independently selected from threonine or serine;

each X_2 is independently selected from methionine or cysteine;

each X_3 is independently selected from arginine, lysine or histidine;

each X_4 is independently selected from proline, phenylalanine or tryptophan;

each X_5 is independently selected from asparagine or glutamine; and

each X_6 is independently selected from leucine, isoleucine, valine or alanine.

11.   A conjugate according to claim 10 wherein the amino acid sequence of Formula I is TMTRPHFHKRQLVLS (SEQ. ID. NO.: 11).

12.   A conjugate according to claim 5 wherein the HA binding peptide

has an amino acid sequence comprising the following Formula II:

$Y_1 - Y_1 - Y_2 - Y_2 - Y_1 - Y_3 - Y_1 - Y_3 - Y_3 - Y_1 - Y_3 - Y_1 - Y_2 - Y_3 - Y_3$

wherein:

each Y_1 is independently selected from a hydroxy amino acid residue;

each Y_2 is independently selected from a sulfur containing amino acid residue; and

each Y_3 is independently selected from a basic amino acid residue;

and preferably:

each Y_1 is independently selected from serine or threonine;

each Y_2 is independently selected from methionine or cysteine; and

each Y_3 is independently selected from arginine, lysine or histidine.

13.   A conjugate according to claim 12 wherein the amino acid sequence of the Formula II is STMMSRSHKTRSCHH (SEQ. ID. NO.: 12).

14.   A conjugate according to claim 5 wherein the HA binding peptide has an amino acid sequence comprising the following Formula III:

$Z_1 - Z_1 - Z_2 - Z_2 - Z_1 - Z_3 - Z_1 - Z_3 - Z_3 - Z_1 - Z_3 - Z_1 - Z_3 - Z_3$

wherein:

each Z_1 is independently selected from a hydroxy amino acid residue;

each Z_2 is independently selected from a sulfur containing amino acid residue; and

each Z_3 is independently selected from a basic amino acid residue, and fragments;

and preferably,

each Z_1 is independently selected from serine or threonine;

each Z_2 is independently selected from methionine or cysteine; and

each Z_3 is independently selected from arginine, lysine or histidine.

15.   A conjugate according to claim 14 wherein the amino acid sequence of the Formula III is STMMSRSHKTRSHH (SEQ.ID.NO.: 13).

16.   A conjugate according to claim 15 wherein a valine residue is

placed at the C-terminal and obtains the following sequence: STMMSRSHKTRSHHV (SEQ. ID. NO.: 14).

17. A conjugate according to any of claims 1-16 wherein the therapeutic agent is selected from the group comprising a colony stimulating factor (G-CSF, GM-CSF or M-CSF), erythropoietin, growth hormone, parathyroid hormone, an interferon, an interleukin, an antibody, an immunoadhesin, or any functional fragment of any of the forgoing.

18. A method of preparing a conjugate according to any one of claims 5-17 comprising the following steps: (i) inserting a first nucleotide sequence encoding a HA binding protein directly linked to a second nucleotide sequence encoding a therapeutic protein into a suitable vector; (ii) expressing the vector in an acceptable host; and (iii) purifying said conjugate molecule from said host or expression medium.

19. A method according to claim 18 wherein the first nucleotide sequence encodes an HA binding peptide.

20. A method according to claim 18 or 19 wherin the first nucleotide sequence is indirectly linked to the second nucleotide sequence.

21. A process for preparing a pharmaceutical for treating an animal in need of treatment comprising the steps of preparing a purified conjugate according to anyone of claims 1-17 and suspending the conjugate molecule in a pharmaceutically acceptable carrier, diluent or excipient.

22. A pharmaceutical composition comprising purified conjugate according to anyone of claims 1-17 suspended in a pharmaceutically acceptable carrier, diluent, or excipient.

23. A method for altering in vivo the distribution of a therapeutic agent comprising conjugating the therapeutic agent to form a conjugate according to any one of claims 1-17, administering the conjugate to the

animal where the conjugate molecule will distribute primarily in tissues and organs containing high levels of endogenous HA.

24.   A method of treating a mammal with a disorder where a diseased tissue/organ of the mammal contains high levels of HA comprising administering a conjugate according to anyone of claims 1-17 to said mammal.

SEQUENCE LISTING

Information for SEQ ID NO.: 1:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 725 amino acids
(B) Type: Amino acids
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Polypeptide

(vi)   ORIGINAL SOURCE:
(A)   ORGANISM: Human

(vii)   IMMEDIATE SOURCE:
        (B)   CLONE:

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
1      msfpkaplkr fndpsgcaps pgaydvktle vlkgpvsfqk sqrfkqqkes kqnlnvdkdt
61     tlpasarkvk sseskkesqk ndkdlkilek eirvllqerg aqdrriqdle telekmearl
121    naalrektsl sannatlekq lieltrtnel lkskfsengn qknlrilsle lmklrnkret
181    kmrgmmakqe gmemklqvtq rsleesqgki aqlegklvsi ekekidekse teklleyiee
241    iscasdqvek ykldiaqlee nlkekndeil slkqsledni vilskqvedl nvkcqllete
301    kedhvnrnre hnenlnaemq nleqkfileq reheklqqke lqidsllqqe kelsssslhqk
361    lcsfqeemvk eknlfeeelk qtldeldklq qkeeqaerlv kqleeeaksr aeelklleek
421    lkgkeaelek ssaahtqatl llqekydsmv qsledvtaqf esykaltase iedlklenss
481    lqekaakagk naedvqhqil atessnqeyv rmlldlqtks alketeikei tvsflqkitd
541    lqnqlkqqee dfrkqledee grkaekentt aelteeinkw rllyeelynk tkpfqlqlda
601    fevekqalln ehgaaqeqln kirdsyakll ghqnlkqkik hvvklkdens qlksevsklr
661    cqlakkkqse tklqeelnkv lgikhfdpsk afhheskenf alktplkegn tncyrapmec
721    qeswk
```

Information for SEQ ID NO.: 2:

(iii) SEQUENCE CHARACTERISTICS
(E) LENGTH: 794 amino acids
(F) Type: Amino acids
(G) STRANDEDNESS:
(H) TOPOLOGY: Linear

(iv) MOLECULE TYPE: Polypeptide

(vi)   ORIGINAL SOURCE:

(A)   ORGANISM: Mus musculus (mouse)

(vii)   IMMEDIATE SOURCE:
        (B)   CLONE:

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
1      msfpkaplkr fndpsgcaps pgaydvktse atkgpvsfqk sqrfknqres qqnlsidkdt
61     tllasakkak ksvskkdsqk ndkdvkrlek eirallqerg tqdkriqdme selekteakl
121    naavrektsl sasnaslekr lteltranel lkakfsedgh qknmralsle lmklrnkret
181    kmrsmmvkqe gmelklqatq kdlteskgki vqlegklvsi ekekidekce teklleyiqe
241    iscasdqvek ckvdiaqlee dlkekdreil slkqsleeni tfskqiedlt vkcqlleter
301    dnlvskdrer aetlsaemqi lterlalerq eyeklqqkel qsqsllqqek elsarlqqql
361    csfqeemtse knvfkeelkl alaeldavqq keeqserlvk qleeerksta eqltrldnll
421    rekevelekh iaahaqaili aqekyndtaq slrdvtaqle svqekyndta qslrdvtaql
481    eseqekyndt aqslrdvtaq leseqekynd taqslrdvta qlesvqekyn dtaqslrdvs
541    aqlesyksst lkeiedlkle nltlqekvam aeksvedvqq qiltaestnq eyarmvqdlq
601    nrstlkeeei keitssflek itdlknqlrq qdedfrkqle ekgkrtaeke nvmteltmei
661    nkwrllyeel yektkpfqqq ldafeaekqa llnehgatqe qlnkirdsya qllghqnlkq
721    kikhvvklkd ensqlksevs klrsqlvkrk qnelrlqgel dkalgirhfd pskafchask
781    enftplkegn pncc
```

Information for SEQ ID NO.: 3:

(v) SEQUENCE CHARACTERISTICS
(I) LENGTH: 713 amino acids
(J) Type: Amino acids
(K) STRANDEDNESS:
(L) TOPOLOGY: Linear

(vi) MOLECULE TYPE: Polypeptide

(vi)   ORIGINAL SOURCE:
(A)   ORGANISM: Rattus norvegicus (rat)

(vii) IMMEDIATE SOURCE:
       (B)   CLONE:

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
1      msfpkaplkr fndpsgcaps pgaydvktse stkgpvsfqk sqrfknqres qqnlnidkdt
61     tllasakkak tlvskkesqk ndkdvkrlek eihvllqerg tqdkriqdme selenteanl
121    napvtekpsl sannaslekr lteltranel lkskfsedah qknmrtlsle lmklrnkret
181    kmrsmmakqe gmelklqatq kdllieskgki vqlegklvsi ekekidekse teklleyiee
241    iscasdqvek ykldiaqlee dlkekdreil clkqsleekv sfskqiedlt vkcqlleaer
301    ddlvskdrer aeslsaemqv lteklllerq eyeklqqnel qsqsllqqek elsahlqqql
361    csfqeemtse rnvfkeqlkl aldeldavqq kkeqseklvk qleeetksta eqlrrlddll
```

```
421    rekeielekr  taahaqatvi  aqekysdtaq  tlrdvtaqle  syksstlkei  edlklenltl
481    qekvamaekr  vedvqqqilt  aestnqeyak  vvqdlqnsst  lkeaeikeit  ssylekitdl
541    qnqlrqqned  frkqleeega  kmteketavt  eltmeinkwr  llyeelfdkt  kpfqqqldaf
601    eaekqallne  hgatqeqlsk  irdsyaqllg  hqnlkqkikh  vvklkdensq  lksevsklrs
661    qlakrkqnel  rlqgeldkal  girhfdpska  fcheskenvt  lktplkegnp  ncc
```

Information for SEQ ID NO.: 4:

(vii) SEQUENCE CHARACTERISTICS
(M) LENGTH: 3114 base pairs
(N) Type: cDNA
(O) STRANDEDNESS:
(P) TOPOLOGY: Linear

(viii) MOLECULE TYPE: Bases

(vi)   ORIGINAL SOURCE:
(A)   ORGANISM: Human

(vii)   IMMEDIATE SOURCE:
        (B)   CLONE:

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
1      ccgccagtgt  gatggatatc  tgcagaattc  ggcttactca  ctatagggct  cgagcggccg
61     cccgggcagg  tgtgccagtc  accttcagtt  tctggagctg  gccgtcaaca  tgtcctttcc
121    taaggcgccc  ttgaaacgat  tcaatgaccc  ttctggttgt  gcaccatctc  caggtgctta
181    tgatgttaaa  actttagaag  tattgaaagg  accagtatcc  tttcagaaat  cacaaagatt
241    taaacaacaa  aaagaatcta  aacaaatct   taatgttgac  aaagatacta  ccttgcctgc
301    ttcagctaga  aaagttaagt  cttcggaatc  aaagaaggaa  tctcaaaaga  atgataaaga
361    tttgaagata  ttagagaaag  agattcgtgt  tcttctacag  gaacgtggtg  cccaggacag
421    gcggatccag  gatctggaaa  ctgagttgga  aaagatggaa  gcaaggctaa  atgctgcact
481    aagggaaaaa  acatctctct  ctgcaaataa  tgctacactg  gaaaacaac   ttattgaatt
541    gaccaggact  aatgaactac  taaaatctaa  gttttctgaa  aatggtaacc  agaagaattt
601    gagaattcta  agcttggagt  tgatgaaact  tagaaacaaa  agagaaacaa  agatgagggg
661    tatgatggct  aagcaagaag  gcatggagat  gaagctgcag  gtcacccaaa  ggagtctcga
721    agagtctcaa  gggaaaatag  cccaactgga  gggaaaactt  gtttcaatag  agaaagaaaa
781    gattgatgaa  aaatctgaaa  cagaaaaact  cttggaatac  atcgaagaaa  ttagttgtgc
841    ttcagatcaa  gtggaaaaat  acaagctaga  tattgcccag  ttagaagaaa  atttgaaaga
901    gaagaatgat  gaaattttaa  gccttaagca  gtctcttgag  gaaaatattg  ttatattatc
961    taaacaagta  gaagatctaa  atgtgaaatg  tcagctgctt  gaaaagaaa   aagaagacca
1021   tgtcaacagg  aatagagaac  acaacgaaaa  tctaaatgca  gagatgcaaa  acttaaaaca
1081   gaagtttatt  cttgaacaac  aggaacatga  aaagcttcaa  caaaagaat   tacaaattga
1141   ttcacttctg  caacaagaga  aagaattatc  ttcgagtctt  catcagaagc  tctgttcttt
1201   tcaagaggaa  atggttaaag  agaagaatct  gtttgaggaa  gaattaaagc  aaacactgga
1261   tgagcttgat  aaattacagc  aaaaggagga  acaagctgaa  aggctggtca  agcaattgga
```

```
1321   agaggaagca aaatctagag ctgaagaatt aaaactccta gaagaaaagc tgaaagggaa
1381   ggaggctgaa ctggagaaaa gtagtgctgc tcatacccag gccaccctgc ttttgcagga
1441   aaagtatgac agtatggtgc aaagccttga agatgttact gctcaatttg aaagctataa
1501   agcgttaaca gccagtgaga tagaagatct taagctggag aactcatcat tacaggaaaa
1561   agcggccaag gctgggaaaa atgcagagga tgttcagcat cagatttggg caactgagag
1621   ctcaaatcaa gaatatgtaa ggatgcttct agatctgcag accaagtcag cactaaagga
1681   aacagaaatt aaagaaatca cagtttcttt tcttcaaaaa ataactgatt gcagaaccca
1741   actcaagcaa caggaggaag actttagaaa acagctggaa gatgaagaag gaagaaaagc
1801   tgaaaaagaa aatacaacag cagaattaac tgaagaaatt aacaagtggc gtctcctcta
1861   tgaagaacta tataataaaa caaaaccttt tcagctacaa ctagatgctt ttgaagtaga
1921   .aaaacaggca ttgttgaatg aacatggtgc agctcaggaa cagctaaata aaataagaga
1981   ttcatatgct aaattattgg gtcatcagaa tttgaaacaa aaaatcaagc atgttgtgaa
2041   gttgaaagat gaaaatagcc aactcaaatc ggaagtatca aaactccgct gtcagcttgc
2101   taaaaaaaaa caaagtgaga caaaacttca agaggaattg aataaagttc taggtatcaa
2161   acactttgat ccttcaaagg cttttcatca tgaaagtaaa gaaaattttg ccctgaagac
2221   cccattaaaa gaaggcaata caaactgtta ccgagctcct atggagtgtc aagaatcatg
2281   gaagtaaaca tctgagaaac ctgttgaaga ttatttcatt cgtcttgttg ttattgatgt
2341   tgctgttatt atatttgaca tgggtatttt ataatgttgt atttaatttt aactgccaat
2401   .ccttaaatat gtgaaaggaa catttttttac caaagtgtct tttgacattt tattttttct
2461   tgcaaatacc tcctccctaa tgctcacctt tatcacctca ttctgaaccc tttcgctggc
2521   tttccagctt agaatgcatc tcatcaactt aaaagtcagt atcatattat tatcctcctg
2581   ttctgaaacc ttagtttcaa gagtctaaac cccagattct tcagcttgat cctggaggct
2641   tttctagtct gagcttcttt agctaggcta aaacaccttg gcttgttatt gcctctactt
2701   tgattcttga taatgctcac ttggtcctac ctattatcct ttctacttgt ccagttcaaa
2761   taagaaataa ggacaagcct aacttcatag taacctctct attttaatca gttgtttaat
2821   aatttacagg ttcttaggct ccatcctgtt tgtatgaaat tataatctgt ggattggcct
2881   ttaagcctgc attcttaaca aactcttcag ttaattctta gatacactaa aaatctgaag
2941   aaactctaca tgtaactatt tcttcagagt ttgtcatata ctgcttgtca tctgcatgtc
3001   tactcagcat ttgattaaca tttgtgtaat aagaaataaa attacacagt aagtcattta
3061   accaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaa
```

Information for SEQ ID NO.: 5:

(ix) SEQUENCE CHARACTERISTICS
(Q) LENGTH: 2479 base pairs
(R) Type: mRNA
(S) STRANDEDNESS:
(T) TOPOLOGY: Linear

(x) MOLECULE TYPE: Bases

(vi)   ORIGINAL SOURCE:
(A)   ORGANISM: Mouse

(vii)   IMMEDIATE SOURCE:
(B)   CLONE:

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
1      ctgtctcatc tggacccagg cgtcagaatg tcctttccta aggcgcccct gaagagattc
61     aatgaccctt cgggttgtgc tccatctccg ggtgcttatg atgttaaaac ttcagaagca
121    actaaaggac cagtgtcttt tcagaaatca caaagattta aaaaccaaag agagtctcaa
181    caaaatctta acattgacaa agatacaacc ttgcttgctt cggctaaaaa agcaaagacg
241    tctgtgtcaa agaaggactc tcagaagaat gataaagatg tgaagagatt acaaaaacac
301    attcgcgctc ttttgcaaga gcgagggact caggacaaac ggatccagga catggaatct
361    gaattggaga agacagaagc aaagctcaat gcagcagtca gagagaaaac atctctctct
421    gcgagtaatg cttcactgga aaaacggctt actgaattaa ccagagccaa cgagctacta
481    aaggctaagt tttctgaaga tggtcaccaa aagaatatga gagctctaag cctggaattg
541    atgaaactca gaaataagag agagacaaag atgaggagta tgatggtcaa acaggaaggc
601    atggagctga agctgcaggc cactcagaag gacctcacgg agtctaaggg aaaaatagtc
661    cagctggagg gaaagcttgt ttcaatagag aaagaaaaga tcgatgaaaa atgtgaaaca
721    gaaaaactct tagaatacat ccaagaaatt agctgtgcat ctgatcaagt ggaaaaatgc
781    aaagtagata ttgcccagtt agaagaagat ttgaaagaga aggatcgtga gattttaagt
841    cttaagcagt ctcttgagga aaacattaca ttttctaagc aaatagaaga cctgactgtt
901    aaatgccagc tacttgaaac agaaagagac aaccttgtca gcaaggatag agaaagggct
961    gaaactctca gtgctgagat gcagatcctg acagagaggc tggctctgga aaggcaagaa
1021   tatgaaaagc tgcaacaaaa agaattgcaa agccagtcac ttctgcagca agagaaggaa
1081   ctgtctgctc gtctgcagca gcagctctgc tctttccaag aggaaatgac ttctgagaag
1141   aacgtcttta agaagagct aaagctcgcc ctggctgagt tggatgcggt ccagcagaag
1201   gaggagcaga gtgaaaggct ggttaaacag ctggaagagg aaaggaagtc aactgcagaa
1261   caactgacgc ggctggacaa cctgctgaga gagaagaag ttgaactgga gaaacatatt
1321   gctgctcacg cccaagccat cttgattgca caagagaagt ataatgacac agcacagagt
1381   ctgagggacg tcactgctca gttggaaagt gtgcaagaga agtataatga cacagcacag
1441   agtctgaggg acgtcactgc tcagttggaa agtgagcaag agaagtacaa tgacacagca
1501   cagagtctga gggacgtcac tgctcagttg gaaagtgagc aagagaagta caatgacaca
1561   gcacagagtc tgagggacgt cactgctcag ttggaaagtg tgcaagagaa gtacaatgac
1621   acagcacaga gtctgaggga cgtcactgct cagttggaaa gctataagtc atcaacactt
1681   aaagaaatag aagatcttaa actggagaat ttgactctac aagaaaaagt agctatggct
1741   gaaaaaagtg tagaagatgt tcaacagcag atattgacag ctgagagcac aaatcaagaa
1801   tatgcaagga tggttcaaga tttgcagaac agatcaacct aaaagaaga agaaattaaa
1861   gaaatcacat cttcatttct tgagaaaata actgatttga aaaatcaact cagacaacaa
1921   gatgaagact ttaggaagca gctggaagag aaaggaaaaa gaacagcaga gaaagaaaat
1981   gtaatgacag aattaaccat ggaaattaat aaatggcgtc tcctatatga tgaactatat
2041   gaaaaaacta aacctttca gcaacaactg gatgcctttg aagccgagaa acaggcattg
2101   ttgaatgaac atggtgcaac tcaggagcag ctaaataaaa tcagagactc ctatgcacag
2161   ctacttggtc accagaacct aaagcaaaaa atcaaacatg ttgtgaaatt gaaagatgaa
2221   aatagccaac tcaaatcgga ggtgtcaaaa ctccgatctc agcttgttaa aaggaaacaa
2281   aatgagctca gacttcaggg agaattagat aaagctctgg gcatcagaca ctttgaccct
2341   tccaaggctt tttgtcatgc atctaaggag aattttactc cattaaaaga aggcaaccca
2401   aactgctgct gagttcagat gcaacttcaa gaatcatgga agtatacgtc tgaaatactt
2461   gttgaagatt atttttctt
```

Information for SEQ ID NO.: 6:

(xi) SEQUENCE CHARACTERISTICS
(U) LENGTH: 2286 base pairs
(V) Type: mRNA
(W) STRANDEDNESS:
(X) TOPOLOGY: Linear

(xii) MOLECULE TYPE: Bases

(vi)   ORIGINAL SOURCE:
(A)   ORGANISM: Rat

(vii)   IMMEDIATE SOURCE:
    (B)   CLONE:

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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121   aaaggaccag  tgtcttttca  gaaatcacaa  agatttaaaa  accaaagaga  gtctcaacaa
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601   gagctgaagc  tgcaggccac  tcagaaggac  ctcattgagt  ctaaggggaa  aatagtacag
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1741  accatggaaa  ttaataaatg  gcgtctccta  tatgaagaac  tgtttgacaa  aactaaacct
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1981 tcggaggtgt caaaactccg atctcagctt gctaaaagga aacaaaatga gctcagactt
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2161 tgctgagtca gactgcaggg accgtggaag tggacgtcca agatacttgc tgaagattgt
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2281 actgcc
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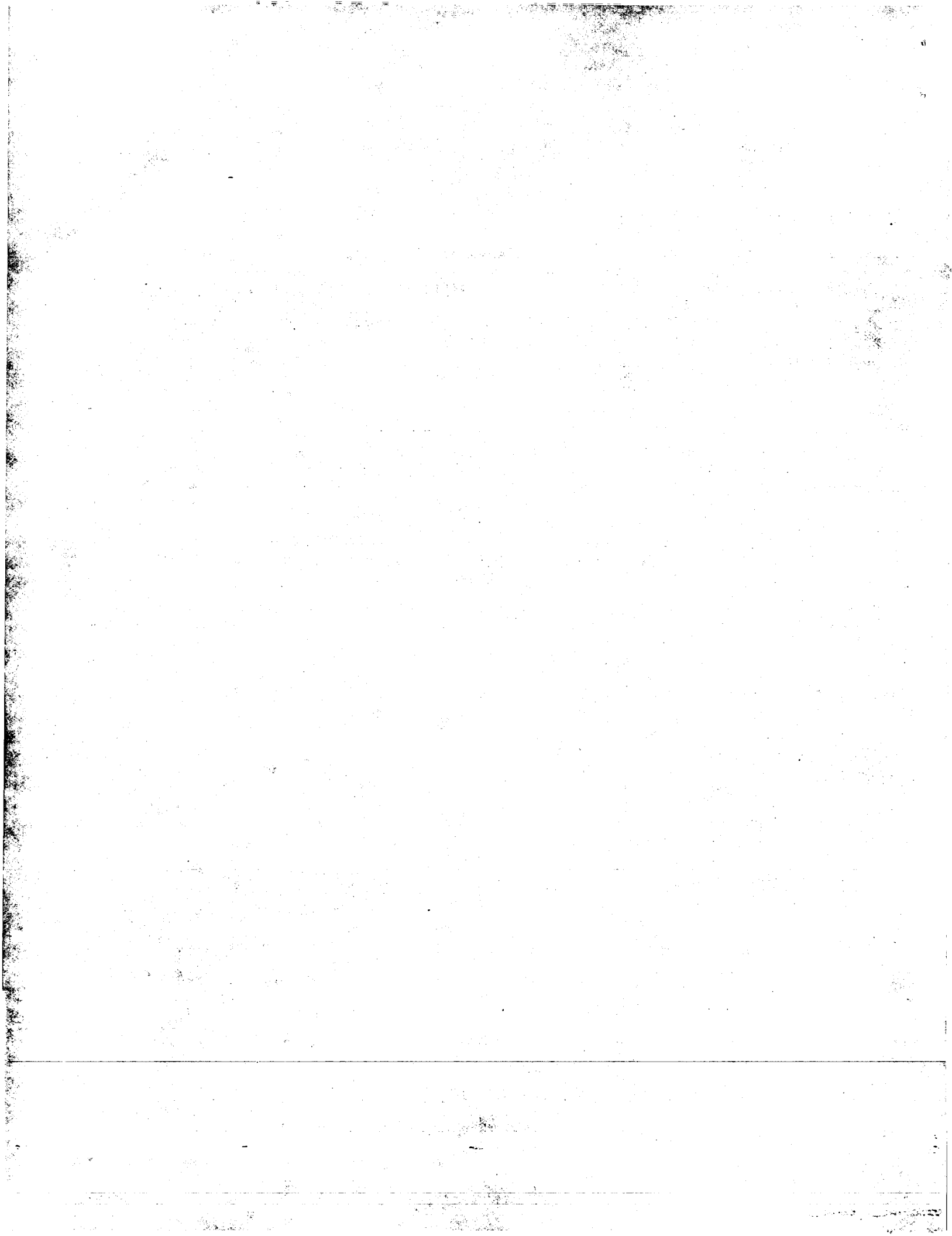
(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 November 2001 (01.11.2001)

PCT

(10) International Publication Number
WO 01/080899 A3

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(25) Filing Language: English

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(71) Applicant (for all designated States except US): CAN-GENE CORPORATION [CA/CA]; 26 Henlow Bay, Winnipeg, Manitoba R3Y 1G4 (CA).

(72) Inventors; and
(75) Inventors/Applicants (for US only): WOLOSKI, B., Michael, R. [CA/CA]; 8 Everden Road, Winnipeg, Manitoba R3N 3Y2 (CA). WILLIAMS, Ashley, Martin [CA/CA]; 14 Branson Crescent, Winnipeg, Manitoba R3T 5W2 (CA). SEREDA, Terrance, Jimmy [CA/CA]; #214-1720 Pembina Highway, Winnipeg, Manitoba R3T 5T4 (CA). WIEBE, Deanna, June [CA/CA]; 528 Raglan Road, Winnipeg, Manitoba R3G 3E5 (CA).

(74) Agent: BERESKIN & PARR; 40th Floor, 40 King Street West, Toronto, Ontario M5H 3Y2 (CA).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— with international search report
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

(88) Date of publication of the international search report:
6 September 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: RHAMM PEPTIDE CONJUGATES

(57) Abstract: The present invention provides protein conjugates having a glucose-aminoglycan-targeting domain conjugated directly or indirectly to a therapeutically useful protein via chemical or peptidyl linkage. The protein conjugates selectively target certain tissues and organs and are useful for treating or preventing various physiological and pathological conditions. Methods of their use and preparation are described.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 950 708 A (CANGENE CO) 20 October 1999 (1999-10-20) cited in the application claims 1-26	1-23
A	WO 93 21312 A (UNIVERSITY OF MANITOBA ET AL) 28 October 1993 (1993-10-28) cited in the application claims 1-44	1-23
A	WO 96 06622 A (HYAL PHARMACEUTICAL CORPORATION) 7 March 1996 (1996-03-07) claims 1-18	1-23
A	WO 98 52590 A (HYAL PHARMACEUTICAL CORPORATION) 26 November 1998 (1998-11-26) cited in the application claims 1-18	1-23

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| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 July 2002	09/07/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Siatou, E

Form PCT/ISA/210 (second sheet) (July 1992)

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	FIEBER C ET AL: "Characterisation of the murine gene encoding the intracellular hyaluronan receptor IHABP (RHAMM)" GENE, ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, NL, vol. 226, no. 1, 8 January 1999 (1999-01-08), pages 41-50, XP004154469 ISSN: 0378-1119 abstract	1-23
A	ASSMANN V ET AL: "The human hyaluron receptoer RHAMM is expressed as an intracellular protein in breast cancer cells" JOURNAL OF CELL SCIENCE, ESSEX, GB, vol. 111, 1998, pages 1685-1694, XP002173591 abstract	1-23
A	CHAO W ET AL: "The characterization of a human RHAMM cDNA: conservation of the hyaluronan-binding domains" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES, ELSEVIER SCIENCE PUBLISHERS, BARKING, GB, vol. 174, no. 2, 1996, pages 299-306, XP004043279 ISSN: 0378-1119 abstract	1-23
A	SAMUEL S K ET AL: "TGF-BETA1 STIMULATION OF CELL LOCOMOTION UTILIZES THE HYALURONAN RECEPTOR RHAMM AND HYALURONAN" JOURNAL OF CELL BIOLOGY, ROCKEFELLER UNIVERSITY PRESS, NEW YORK, US, US, vol. 123, no. 3, 1 November 1993 (1993-11-01), pages 749-758, XP000578044 ISSN: 0021-9525 abstract	1-23
A	YANG B ET AL: "IDENTIFICATION OF A COMMON HYALURONAN BINDING MOTIF IN THE HYALURONAN BINDING PROTEINS RHAMM, CD44 AND LINK PROTEIN" EMBO JOURNAL, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 13, no. 2, 15 January 1994 (1994-01-15), pages 286-296, XP000647316 ISSN: 0261-4189 abstract	1-23

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-5, 7 and 21-24 relate to an extremely large number of possible compounds, compositions containing them, methods for preparing and uses thereof. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds, compositions and methods claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the conjugates comprising
a.) the HA-binding agent is RHAMM of seq. ID 1-3, anpeptide of seq. IDs 7-14 and an anti-HA antibody and
b.) the therapeutic agent is selected from the proteins/peptides listed in claim 17 and the compounds disclosed in examples 7-9 of the present application.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Although claims 23-24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

   see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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